Evaluation of the role of mitochondrial citrate synthase, mitochondrial and cytosolic isoforms of isocitrate dehydrogenase in tomato leaf metabolism

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Eidesstattliche Erklärung

This Ph.D. thesis is the result of my own work and was done between January 2004 and March 2007 in the department of Prof. Dr. Lothar Willmitzer at the Max-Planck-Institute of Molecular Plant Physiology in Golm, Germany. It has not been submitted for any degree or Ph.D. at any other university.

Die Dissertation ist das Ergebnis praktischer Arbeit, welche von Januar 2004 bis März 2007 durchgeführt wurde im Department von Prof. Dr. Lothar Willmitzer im Max-Planck-Insitut für Molekulare Pflanzenphysiologie, Golm, Deutschland. Ich versichere, daß ich die vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Diese Dissertation wurde an keiner anderen Hochschule zu Prüfung eingereicht.

Berlin, September 2009

Agata Sienkiewicz-Porzucek

Zusammenfassung

Der Citratzyklus (TCA) ist einer der bedeutendsten Stoffwechselwege für alle lebenden Organismen. Trotz der zentralen Rolle dieses Prozesses im Pflanzenmetabolismus ist er nur relativ wenig untersucht worden. In dieser Arbeit berichte ich über die Produktion und die funktionale Analyse von Tomatenpflanzen *(Solanum lycopersicum)*, die unabhängig eine leicht eingeschränkte Aktivität der mitochondrialen Citrat-Synthase (CS) und zweier Isocitrat-dehydrogenasen (mitochondriale NAD-IDH und cytosolische NADP-ICDH) zeigen.

Pflanzen, die einen Teil der Citrat-Synthase (CS) in einer antisense Orientierung exprimieren, wiesen mehrheitlich keine erkennbare Veränderung eines Wachstumphänotyps auf. Obwohl die photosyntetische Leistung keine Änderungen gezeigt hatte, war die mitochondriale Respiration gestiegen, begleitet von einem reduzierten Kohlenstoff-fluss durch den Citratzyklus. Darüber hinaus waren die CS Pflanzen charakterisiert durch wesentliche Änderungen im Blattmetabolismus, einschließlich eines eingeschränkten Niveaus des photosynthetischen Pigments und Zwischenprodukten des Citratzyklus zusammen mit einer Akkumulation von Nitraten, verschiedenen Aminosäuren und Stärken. Interessanterweise wurde die maximale katalytische Aktivität von einigen im primären C und N Metabolismus beteiligten Enzymen reduziert. Zusammengefasst deuten diese Ergebnisse auf eine Einschränkung der Nitrat-Aufnahme hin. Das mit Hilfe von TOM1 Mikroarrays und quantitativer RT-PCR durchgeführte Transcript-profiling hat gezeigt, dass die fehlende Aktivität der mitochondrialen CS teilweise von einer gestiegenen, peroxisomalen CS Isoform ausgeglichen wird.

Die genetisch modifizierten Tomatenpflanzen, die ein Fragment der Kodierungsregion der mitochondrialen NAD-abhängigen Isocitrat-Dehydrogenase (NAD-IDH) in einer antisense Orientierung exprimieren, und Pflanzen mit einer durch RNAi Ansatz geminderten Aktivität der zytologischen NADP-abhängigen Isocitrat-Dehydrogenase (NADP-ICDH) haben leichte, phänotypische Änderungen gezeigt, meistens in Form von reduziertem Fruchtwachstum. Zusätzlich wurde auch bei NADP-ICDH Pflanzen ein verstärktes Wurzelwachstum festgestellt. Die maximale Effizienz des Photosystems II ist in beiden transgenen Genotypen leicht reduziert worden bei gleichzeitigem, deutlichen Abfall im Niveau des photosynthetischen Chlorophylls und Xantophylls. Trotzdem konnte eine klare Minderung des metabolischen Kohlenstoff-Flusses durch den Citratzyklus und ein reduziertes Niveau der Citratzyklus-Zwischenprodukte ausschliesslich in NAD-IDH Linien beobachtet werden. Zusätzlich wurde in beiden NAD-IDH und NADP-ICDH Pflanzentypen wesentliche metabolische Änderungen entdeckt, wie z.B. eine reduzierte Stärkebiosynthese, Ansammlung von Nitrat und Veränderungen des Aminosäuren-

und Pyridinnukleotidgehaltes. Die metabolische Verschiebung, kombiniert mit Veränderungen des zellularen Niveaus von Reduktionsäquivalenten ergab eine Verstärkung der photorespiratorischen Leistung, die vermutlich eine ausgleichende Rolle in der Produktion organischer Säuren und der Wiederherstellung der Redox-Balance spielt. Interessanterweise zeigen beiden Genotypen verschiedene Symptome von Störungen in der Nitratassimilation auf enzymatischen, metabolischen, transcript und phänotypischen Ebenen auf. Die metabolische Antwort von Blättern auf Stickstoffmangel war in transgenen NADP-ICDH Pflanzen dramatischer als in NAD-IDH Pflanzen, was darauf hindeutet, dass die cytosolische Isoform der Hauptlieferant von 2-Oxoglutarat im Tomatenmetabolismus sein könnte.

Die in dieser Arbeit präsentierten Ergebnisse haben zu einem tieferen Einblick in die funktionale Role der mitochondrialen Citratsynthase und auch der mitochondrialen und cytosolischen Isocitrat-Dehydrogenasen im Blattmetabolismus einer Modelpflanze (*Solanum lycopersicum*) geführt. Sie haben das Bestehen einer zwischenorganellen Koordination des Stoffwechselprozesses hervorgehoben und unser Verständnis der Kohlenstoff-Stickstoff Wechselwirkung erhöht. Desweiteren haben die Ergebnisse die Anwesenheit von Strategien nachgewiesen, durch die der Metabolismus bei auftauchenden Mängeln umprogrammiert werden kann.

Abstract

The TCA cycle is a respiratory metabolic pathway of central importance for all living organisms. Despite the vital function of this process in plant metabolism relatively few molecular physiological studies were performed to date. Here, I report the generation and functional analysis of tomato plants (*Solanum lycopersicum)* independently displaying mildly limited activity of mitochondrial citrate synthase (CS) and two isocitrate dehydrogenases, namely mitochondrial NAD-IDH and cytosolic NADP-ICDH.

The plants expressing a fragment of mitochondrial citrate synthase (CS) in the antisense orientation exhibited essentially no visible growth phenotype. Although photosynthetic performance was unaltered, mitochondrial respiration was increased and accompanied by limited carbon flux through the TCA cycle. Moreover, the CS transgenic plants were characterized by significant modifications in the leaf metabolic content. They included limited level of photosynthetic pigments and TCA cycle intermediates, in addition to accumulation of nitrate, multiple amino acids and starch. Interestingly, the maximal catalytic activities of several enzymes involved in primary C and N metabolism were decreased. When taken together, these results hint towards limitations in nitrate assimilation pathway. The transcript profiling performed by utilizing TOM1 microarrays and quantitative RT-PCR approach revealed that the deficiency in mitochondrial CS activity was partially compensated by upregulation of peroxisomal CS isoform.

The transgenic tomato plants expressing a fragment of mitochondrial NAD-dependent isocitrate dehydrogenase (NAD-IDH) in the antisense orientation and plants down regulated in the activity of cytosolic NADP-dependent isocitrate dehydrogenase (NADP-ICDH) via the RNAi approach revealed minor phenotypic modifications, manifested mainly by compromised fruit production. Additionally, NADP-ICDH plants displayed increased root formation. The maximal efficiency of photosystem II was mildly reduced in both transgenic genotypes and accompanied by significant decrease in the level of photosynthetic chlorophylls and xantophylls. However, a clear reduction in the metabolic carbon flux through the TCA cycle and reduced level of TCA cycle intermediates were observed exclusively in the NAD-IDH transgenic lines. Furthermore, both NAD-IDH and NADP-ICDH plants revealed large metabolic alterations, such as decreased starch biosynthesis, accumulation of nitrate and modifications in amino acids and pyridine nucleotides content. The metabolic shift combined with modifications in the cellular reducing equivalent level resulted in upregulation of the photorespiratory pathway, which presumably played a compensatory role in supporting organic acid production and re-establishing redox balance. Interestingly, both transgenic genotypes exhibited various symptoms of perturbations in nitrate assimilation on the enzymatic, metabolic, transcript and phenotypic levels. The leaf metabolic response towards nitrogen starvation conditions was far more dramatic in NADP-ICDH transgenic plants

than NAD-IDH plants, hinting that the cytosolic isoform may be the major 2-oxoglutarate supplier in tomato metabolism.

The results obtained in this work provided a deeper insight into of the functional role of mitochondrial citrate synthase and mitochondrial and cytosolic isocitrate dehydrogenases in plant metabolism. Additionally, they highlighted the existence of interorganellar coordination of metabolism and increased our understanding of carbon-nitrogen interactions.

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List of abbreviations

Aco	aconitase
ADP	adenosine diphosphate
ADPglc	adenosine diphosphate glucose
AGPase	adenosine diphosphate glucose pyrophosphorylase
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
bp	base pair
Bq	Becquerel
C	carbon
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CoA	Coenzyme A
CS	citrate synthase
cv	cultivar
DAF	days after flowering
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
Fd	ferredoxin
Fru	fructose
FW	fresh weight
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC-MS	gas chromatography – mass spectrometry
G6PDH	glucose 6-phosphate dehydrogenase
GS	glutamine synthetase
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
Glc	glucose
Gln	glutamine
Glu	glutamate
HPLC	high performance liquid chromatography

IDH/ICDH	isocitrate dehydrogenase
IPMDH	3-isopropylmalate dehydrogenase
kb	kilo bases
LB	Laura Bretani medium
NIA	nitrate reductase
NII	nitrite reductase
MES	2-morpholinoethanesulphonic acid
MDH	malate dehydrogenase
mM	milli molar
mRNA	messenger ribonucleic acid
N	nitrogen
NAD+	oxidised nicotinamid adenine dinucleotide
NADH	reduced nicotinamid adenine dinucleotide
NADP+	oxidised nicotinamid adenine dinucleotide phosphate
NADPH	reduced nicotinamid adenine dinucleotide phosphate
NAD-IDH	mitochondrial NAD-dependent isocitrate dehydrogenase
NADP-ICDH	NADP-dependent isocitrate dehydrogenase
NR	nitrate reductase
NiR	nitrite reductase
2-OG	2-oxoglutarate (2-oxoglutarate)
OD	optical density
OPA	ortho-phthaldialdehyde
P	phosphate
PAM	pulse amplitude modulation
PCR	polymerase chain reaction
PEPcase	phosphoenolpyruvate carboxylase
3-PGA	3-phosphoglyceric acid
PFK	phosphofructokinase
PGI	phosphoglucose isomerase
PGM	phosphoglucomutase
Pi	inorganic phosphate
PK	pyruvate kinase
PPase	pyrophosphatase
PPi	pyrophosphate
PS	photo system
qRT-PCR	quantitative reverse transcription PCR
RNA	ribonucleic acid

RNAi	RNA interference
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
RT-RT-PCR	real time reverse transcription PCR
Rubisco	ribulose-1,5-biphosphate carboxylase/oxygenase
SCoAL	succinyl CoA ligase
SE	standard error
SPP	sucrose phosphate phosphatase
SPS	suscrose phosphate synthase
Suc	sucrose
TCA	cycle tricarboxylic acid cycle
TF(s)	Transcription Factor(s)
UBQ	ubiquitin
UDP	uridine diphosphate
UDPglc	uridine diphosphate glucose
UGPase	uridine diphosphate glucose pyrophosphorylase
UTP	uridine triphosphate
v/v	volume per volume
w/v	weight per volume
WT	wild type

1 GENERAL INTRODUCTION

1.1 Tomato as a plant model system

Tomato *(Solanum lycopersicum)* is a herbaceous, sprawling plant belonging to the Solanaceae or nightshade family. This large and extremely diverse plant family contains over 3000 species with origins in both the Old (eggplant – China, India) and the New World (pepper/tomato/potato – Central and South America) (Knapp, 2002). Due to great economic and nutritional value, more than 18 species of Solanaceae plants are currently utilized in agriculture, among which consumable vegetable crops provide important dietary sources of vitamin A and C and antioxidants, such as lycopene (Palozza and Krinsky, 1992; Mayne, 1996; Bramley, 2000). Although the genomic size of tomato, potato, pepper, and eggplant is not equal (about 950 Mb, 1800 Mb, 3000 Mb, and 1100 Mb respectively), the genetic content and basic chromosome number (x=12) remains similar for many species of Solanaceae family (Livingstone, et al., 1999; Doganlar, et al., 2002).

Tomato plants *(S. lycopersicum)* are native to Central, South and southern North America and presumably originated from a little yellow fruit variety, an ancestor of *S. lycopersicum var. cerasiforme*. Initially, it was grown by the Aztecs in Mexico who called it 'xitomatl' meaning 'plump thing with a navel', until it was named 'tomati' by other Central American tribes. Subsequently, the domestication of wild cherry type of tomato spread to Europe and through the process of selection eventually led to large fruited varieties (Frary, et al., 2000). In 1544 Pietro Andrea Mattioli, an Italian physician and botanist, named tomato a 'pomi d'oro' meaning golden apple, although he considered this plant unhealthy and inedible due to its phenotypic similarity to deadly nightshade *(Atropus belladonna).* Finally, in the 18th century Carl Linnaeus named the tomato *Lycopersicon esculentum,* meaning 'edible wolf peach' as this plant was a major food of wild canids in South America.

Tomato plants belong to crops of world-wide agronomical importance. More than 125 tons of tomato fruits are produced in the world today with China being the largest producer, followed by the United States and Turkey. The chemical composition of fruits is the major quality trait of nutritional and organoleptic value, being relevant for both salad market and processing industry. Since the total soluble solids content, expressed as a Brix index, depends on organic acids and sugar level, it is obvious that the taste of agricultural products is strongly dependent on the performance of plant metabolism. The effect of the TCA cycle activity on the biosynthesis of organic acids has been studied in several important agronomic species. The accumulation of some metabolites, particularly citrate and malate in fruit flesh was found to be regulated during fruit development and affect strongly fruit acidity (Etienne, et al., 2002).Although the activity of citrate synthase (CS) and isocitrate dehydrogenase enzymes are believed to have a great impact on fruit

quality, other metabolic pathways have also been modified in order to enrich tomato flavor (Davidovich-Rikanati, et al., 2007). Apart from utilization for consumption, the fruit production of tomato is very valuable for scientific research. The significant size and weight of plants and ability to generate big, fleshy fruits are important features that simplify investigation of development, maturation, ripening, and associated quality and yield traits. Similarly to fruits of cucurbits, avocado, banana, peaches, plums and apples, tomato fruits (botanically: berries) are considered climacteric because they possess increased rates of respiration and ethylene biosynthesis (Giovannoni, 2001). Moreover, tomatoes exhibit the transition of photosynthetic to respiratory metabolism during fruit ripening. Initially photosynthetically active chloroplasts that are able to fix carbon in fruit tissue differentiate into chromoplasts, a non-photosynthetic plastids. Many studies focus therefore on tomato fruit ripening at the transcript level (Bartley and Ishida, 2002; Fei, et al., 2004; Alba, et al., 2005) aiming to understand the regulation of the transition.

Interestingly, tomato was one of the first plants that were genetically modified using recombinant DNA techniques (Fillatti, et al., 1987). It is the most intensively studied genome of the *Solanaceae* family, encoding approximately 35 000 genes corresponding to less than 25 percent (220-250 Mb) of the total DNA in the nucleus. It provides the smallest diploid genome for which homozygous inbred lines are available. In order to facilitate positional cloning of tomato and other *Solanaceae* genomes (via synteny maps) an 'International Solanaceae Genomics Project (SOL): Systems Approach to Diversity and Adaptation' (http://www.sgn.cornell.edu/solanaceae-project/index.pl) was initiated. Since November 2003 an international consortium of 10 countries (Korea, China, the United Kingdom, India, The Netherlands, France, Japan, Spain, Italy and the United States) is responsible for the sequencing of BAC clones derived from a single, common L. esculentum x L. pennellii F2 population by tiling path method. However, huge amount of sequenced cDNA clones of genes expressed in tomato is already publicly available. Expressed Sequence Tags (ESTs) were created by sequencing the 5´and/or 3´ends of randomly isolated gene transcripts that have been converted into cDNA (Adams, et al., 1991). The tomato EST collection was obtained by Clemson University Genomics Centre (http://www.genome.clemson.edu) and organized into a public database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato). It was first described by van der Hoeven and collaborators (Van der Hoeven, et al., 2002) and subsequently utilized for multiple plant studies (Alba, et al., 2004; Fei, et al., 2004; Baxter, et al., 2005; Carbone, et al., 2005; Uppalapati, et al., 2005; Urbanczyk-Wochniak, et al., 2005). Huge amount of data generated by EST sequencing projects and other high-throughput phenotypic technologies are being combined together by the SOL Genomics Network (SGN; http://sgn.cornell.edu). As a part of the International Solanaceae Initiative (SOL) this powerful database was created in order to provide the linkage between phenome and genome of *Solana-*

ceae species, including tomato, potato, tobacco, eggplant, pepper and petunia plants.

1.2 Energy supply and carbon metabolism in plants

1.2.1 Plant metabolic respiration

Respiration in plants, as in all living organisms, is essential to provide metabolic energy and carbon skeletons for growth and development. The conservation of energy during oxidation of substrates is usually performed by coupled synthesis of energy-rich compounds, such as adenosine triphosphate (ATP). In the majority of organisms the synthesis of ATP occurs predominantly in mitochondria, which makes these ubiquitous organelles vital to all eukaryotic life. In addition to the crucial role in ATP generation by oxidative phosphorylation, mitochondria are involved in the production of precursors for a number of essential biosynthetic processes such as nitrogen fixation and the biosynthesis of amino acids, tetrapyrroles, phospholipids, nucleotides and vitamin cofactors. Recently plant mitochondria were shown to be involved in fatty acid synthesis (Gueguen, et al., 2000), synthesis of folate (Mouillon, et al., 2002) and ascorbate (Bartoli, et al., 2000), synthesis and export of iron-sulphur clusters (Kushnir, et al., 2001) and degradation of branched chain amino acids, phytol and lipids (Ishizaki, et al., 2005; Baker, et al., 2006). The core responsibility for essential respiratory processes occurring within mitochondria and complex linkage and interdependence with vital biosynthetic pathways places the mitochondrion in the central position in the metabolic network of eukaryotes. The organelles were identified over fifty years ago as the site of oxidative energy metabolism (Lehninger and Kennedy, 1948), however the basic mechanism that controls mitochondrial shape, size and number has only been discovered recently in yeast (Shaw and Nunnari, 2002).

Due to differences in evolution, as compared to other organisms plants have gained increased flexibility of respiratory pathways (Mackenzie and McIntosh, 1999). The unique features of their metabolism include:

- multiple entry points into the respiratory pathway from sucrose and starch;
- the duplication of pyrophosphate and ATP-dependent phosphorylation of fructose 6-phophate;
- the loss of regulation of glycolysis by kinetic effects of ATP on phosphofructokinase and pyruvate kinase reactions;
- and the presence of non-phosphorylating electron transport systems (Fernie, et al., 2004).

Moreover, a number of specific metabolite exchanges between mitochondria and cytosol have been identified in plants, but not in animals (Douce and Neuburger, 1989; Picault, et al., 2002). These unique adaptations promote survival of plants in

extreme environments and during unavoidable stresses. Apart from 'developmental plasticity' such as increased allocation of biomass to roots that typically occurs in dry or nutrient-deficient conditions, plants has evolved also 'metabolic flexibility' which allows them, in contrast to animals, to frequently accomplish the same step in a metabolic pathway in a variety of different ways (Plaxton and Podesta, 2006). The implementation of metabolic bypasses is best exemplified by genetic engineering experiments, in which antisense elimination or co-suppression of an enzyme traditionally considered to be essential for life resulted in transgenic plants that were able to grow and develop almost normally (Plaxton, 1996; Knowles, et al., 1998). (Dennis and Blakeley, 2000) A central features of plant metabolic flexibility are represented by respiratory metabolism that is typically divided into three main pathways: glycolysis, mitochondrial tricarboxylic (TCA) cycle and mitochondrial electron transport, which functioning and regulation in tightly bound *in vivo.* The energy producing processes are additionally dependent on whole cell metabolism and physiological state. For instance, respiration relies on photosynthesis for production of carbon-rich substrates whereas photosynthesis depends on respiration for generation of energy and carbon intermediates necessary for biosynthesis and growth (Hoefnagel, et al., 1998; Gardestrom, et al., 2002; Raghavendra and Padmasree, 2003; Noctor, et al., 2004; Yoshida, et al., 2006). In keeping with vital role for mitochondria in plant physiology and performance, surprisingly little attention has been paid to investigate these organelles until recent studies (Kruft, et al., 2001; Sweetlove, et al., 2002; Logan, et al., 2003).

1.2.1.1 Glycolysis

Glycolysis was the first major metabolic pathway that became fully elucidated in around 1940. Subsequent studies have shown that it is present, at least in part, in all organisms, although its role, structure, regulation, and localization can show significant differences even within different cells of the same species (Plaxton, 1996). This catabolic anaerobic pathway has evolved in order to fulfill two main functions: oxidation of hexoses to generate ATP, reductants, and pyruvate, and production of building blocks for anabolism. It is an amphibolic pathway, as it can additionally function in the reverse direction by generation of hexoses from low-molecular weight compounds. This gluconeogenic activity is however energy-dependent. In plants, glycolysis is the predominant pathway that fuels plant respiration, in contrast to animal cells which frequently respire fatty acids. Moreover, a significant proportion of the carbon that enters the glycolytic and tricarboxylic acid cycle pathways are utilized for the biosynthesis of multiple compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids.

In the majority of organisms glycolysis is the cytosolic linear sequence of ten enzymatic reactions that catalyze conversion of glucose to pyruvate. Higher plants use sucrose and starch as the principal substrates for glycolysis, which can occur independently in the cytosol and in the plastids. This feature differentiates plants

from other eukaryotes and allows for the interaction between glycolysis intermediates in both compartments through the action of highly selective transporters. Interestingly, the fact that these parallel reactions are catalyzed by distinct nuclear-encoded isozymes combined with the existence of multiple enzymes that bypasses all steps of the plant glycolysis in the two compartments provides the plant with immense metabolic flexibility. The investigation of Arabidopsis mitochondria provided convincing proof that the entire cytosolic glycolytic pathway is intimately and functionally associated with the outer mitochondrial membrane of plant mitochondria (Giege, et al., 2003). This microcompartmentation of glycolysis would facilitate direct provision of pyruvate to the mitochondria to be used as respiratory substrate. Recent studies have supported the theory of dual localization of glycolysis and its close, dynamic interaction with mitochondria by showing that the extent of mitochondrial association of glycolytic enzymes is dependent on respiration rate in both Arabidopsis cells and potato tubers (Graham, et al., 2007). The authors suggest that competition for intermediates between glycolysis and mitochondrial respiration is presumably regulated by substrate channeling.

Glycolysis is additionally linked to the oxidative pentose phosphate pathway (OPPP) and the predominant route of carbohydrate oxidation depends on the varying requirements for hexose phosphates, reducing power and energy of the cells. Under the majority of conditions the rate of glycolysis is approximately four-fold higher than oxidative pentose phosphate pathway. The main role of the OPPP pathway is to generate the reduced cofactor NADPH and precursors for many biosynthetic pathways including shikimate acid and nucleotide biosynthesis (Debnam, et al., 2004). Additionally, it is also a major pathway involved in the sugar induction of NO_3, NH_4 and SO_4 transporters in roots. Recent investigation of signaling processes in Arabidopsis revealed the existence of OPPP-dependent sugar sensing pathway that governs the regulation of root nitrate and sulfur acquisition by the carbon status of the plant, to coordinate the availability of these three elements for amino acid synthesis (Lejay, et al., 2008).

1.2.1.2 TCA cycle

The tricarboxylic acid (TCA, Figure 1) cycle, also known as the Krebs cycle and citric acid cycle is the central metabolic pathway for all aerobic processes in living organisms. This second stage of cellular respiration was first discovered in pigeon muscle tissue in 1937 by Krebs and Johnson (1937) and subsequently was shown to occur also in plant cells (Beevers, 1961). During the TCA cycle, the complete oxidation of C2 units of acetyl-CoA derived from carbohydrates and lipids into carbon dioxide and water is accompanied by capturing the released energy as reductive power in the form of NADH and $FADH_2$ and in ATP equivalents. Although the enzymes of the TCA cycle are located within the mitochondrial matrix, the reaction intermediates accumulate in the vacuole and the reductive power is directly fed into the electron transport chain for the oxidative phosphorylation process in the inner mitochondrial membrane. In addition to generation of 15 ATP

equivalents per each metabolized pyruvate molecule, the TCA cycle marks a point of divergence of anabolic pathways by providing precursors utilized in the formation of important cellular constituents such as amino acids, fatty acids, flavonoids, alkaloids and isoprenoids. The TCA cycle is therefore thought to play a vital role in meeting the demand for carbon skeletons imposed by anabolic processes such as amino acid synthesis (Douce and Neuburger, 1989; Mackenzie and McIntosh, 1999) and isoprenoid synthesis (Fatland, et al., 2005), regulation of cellular redox (Scheibe, et al., 2005), nitrogen fixation (Hill, et al., 1992) and the control of C/N balance (Noguchi and Terashima, 2006).

Despite the crucial importance of this pathway in plant metabolism, the precise physiological function of TCA cycle has not been fully elucidated yet (Siedow and Day, 2000). To date multiple genes encoding TCA cycle enzymes in plants have been cloned (Schnarrenberger and Martin, 2002); (Hill, et al., 1992) and several proteins have been isolated (Lancien, et al., 1998; Thelen, et al., 1998; Millar, et al., 1999). Moreover, molecular physiology studies were performed on pyruvate dehydrogenase (Yui, et al., 2003), citrate synthase (Landschutze, et al., 1995b), aconitase (Carrari, et al., 2003a), isocitrate dehydroganse (Lemaitre, et al., 2007), succinyl-CoA ligase (Studart-Guimaraes, et al., 2007), fumarase (Nunes-Nesi, et al., 2007a) and malate dehydrogase (Nunes-Nesi, et al., 2005b). The comprehensive analysis of the TCA cycle function in tomato revealed large range of alterations on the metabolic, transcript and physiological level across all organs of mutant and transgenic plants exhibiting decreased activity of selected TCA cycle enzymes. Interestingly, these studies confirmed strong linkage between respiration and photosynthesis, as the plants exhibited modified plant development, growth and yield (Carrari, et al., 2003a; Nunes-Nesi, et al., 2005b; Nunes-Nesi, et al., 2007a; Studart-Guimaraes, et al., 2007). Additionally, inactivation of initial steps of the cycle was shown to influence flower development and plant fertility (Landschutze, et al., 1995b; Yui, et al., 2003). The inconsistency of phenotypic and metabolic changes observed in various TCA cycle mutants supports suggestions that the tricarboxylic acid cycle of plants (Lancien, et al., 1999), like those of microbial and mammalian (McCammon, et al., 2003; Tian, et al., 2005) systems displays a modular structure, in which different parts of the pathway have diverse metabolic functions and activities.

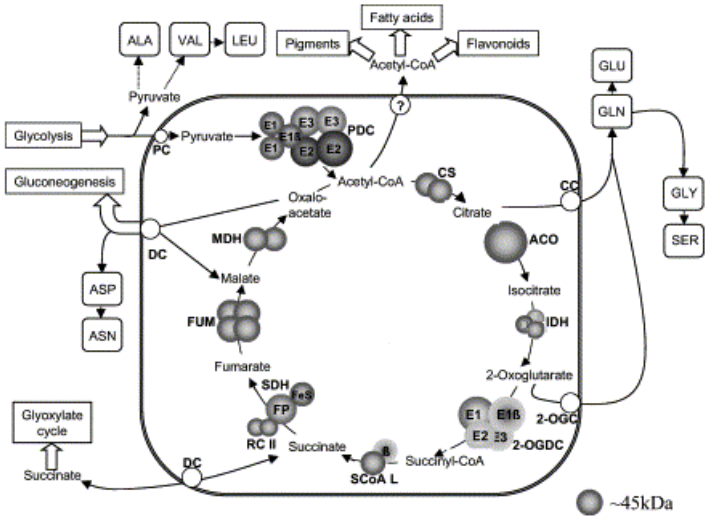


Figure 1: Schematic representation of the TCA cycle and its convergent and divergent pathways. Abbreviations: CS: citrate synthase; ACO: aconitase; IDH: NAD-isocitrate dehydrogenase; 2-OGDC: 2-oxoglutarate dehydrogenase complex; ScoAL: succinyl CoA ligase; SDH: succinate dehydrogenase; FUM: fumarate; MDH: malate dehydrogenase; PDC: pyruvate dehydrogenase complex; PC: pyruvate carrier; DC: dicarboxylate carrier; 2-OGC: 2- oxoglutarate carrier, and CC: citrate carrier.

1.2.1.3 Mitochondrial Electron Transport

The mitochondrial electron chain is responsible for the transfer of electrons, supported by TCA cycle in the form of reducing equivalents to oxygen, the final electron acceptor. The oxidative phosphorylation which occurs during the third step of respiration generates an electrochemical potential which drives ATP synthesis. This energy-rich compound, in addition to NAD and FAD molecules generated by the operation of mitochondrial electron chain are utilized for supporting various metabolic pathways, including TCA cycle. The mitochondrial electron transport is composed of four electron carrier complexes of unique composition. Complexes (I) and (II) catalyses electron transfer to ubiquinone from two different electron donors: NADH (complex I or NADH dehydrogenase complex) and succinate (complex II or succinate dehydrogenase). Complex III (cytochrome bc1 complex or ubiquinone-cytochrome c oxidoreductase) carries electrons from ubiquinone to cytochrome c, and complex IV (cytochrome oxidase) completes the sequence by transferring electrons from cytochrome c to oxygen. The electron transfer through these complexes is tightly coupled to ATP production and is prone to inhibition by cyanide.

Additionally, plants possess unique alternative non-phosphorylating routes for electron transfer, such as internal and external NAD(P)H dehydrogenases and membrane-potential-dissipating uncoupling proteins (Hourton-Cabassa, et al., 2004; Rasmusson, et al., 2004; Vercesi, et al., 2006). The alternative pathway of mitochondrial respiration branches from the cytochrome pathway in the inner mi-

tochondrial membrane at the ubiquinone pool and passes electrons to a single terminal oxidase. This alternative oxidase (AOX), which reduces molecular oxygen to water in a single four-electron transfer step is not coupled to the synthesis of ATP molecules. Moreover, AOX remains cyanide-resistant and is induced by antimycine A, an inhibitor of cytochrome pathway (Millenaar and Lambers, 2003). The AOX enzyme was first discovered in higher plant mitochondria as a thermogenic curiosity observed during anthesis (Skubatz, et al., 1991; Vanlerberghe, et al., 1994) and was recognized as part of a plant's ability to tune the internal energy-carbon balance to the emerging environmental changes (Vanlerberghe, et al., 1995; Watanabe, et al., 2008). It was shown that the AOX is being regulated on both transcriptional and translational levels in response to diverse biotic and abiotic stresses (Ordog, et al., 2002). Interestingly, the activity of this enzyme is also tightly connected to the performance of the TCA cycle. Several major organic amino acids were proven to stimulate the activity of AOX when applied on mitochondria isolated from soybean and potato (for review see (Vanlerberghe and McIntosh, 1997) and more recently from tobacco (Gray, et al., 2004). Additionally, the latter researchers have shown that the tobacco leaves overexpressing mitochondrially located NADP-ICDH contain high level of the activated, reduced form of AOX. It was proposed that depending on the metabolic and energetic balance of plant mitochondria, the respiration operates through NAD-IDH enzyme of the TCA cycle and mitochondrial transport chain in favorable conditions or, alternatively is directed through NADP-ICDH and AOX pathway. Presumably, the thioredoxin/thioredoxin reductase is the critical component connecting the mitochondrial NAD/NADP status and AOX activity (Moller and Rasmusson, 1998), although this statement needs to be experimentally proven yet. The increased respiration through AOX pathway was also observed in Arabidopsis seedling under ammonium nutrition (Escobar, et al., 2006). Sspecifically, the switch from nitrate to ammonium feeding regime resulted in substantial elevation of external NADH oxidation, combined with increased capacity and protein abundance of AOX. The authors proposed that alternative respiration may function in maintaining the redox homeostasis in response to variation in nitrogen sources available in the soil. In general, the alternative respiratory components play a various physiological roles, including thermogenesis (Siedow and Day, 2000), the prevention of reactive oxygen species formation (Moller, 2001; Fernie, et al., 2004) and the dissipation of excess redox equivalents (Raghavendra and Padmasree, 2003). Recent studies of Arabidopsis AOX1A mutant provided interesting information of the connections between AOX and other metabolic pathways, including photosynthesis and photorespiration.

1.2.2 Role of energy metabolism in the illuminated leaf

The mitochondrial respiration is a process of vital importance for survival of all living organisms. Through the pathways of TCA cycle and mitochondrial electron transfer chain, the carbohydrates, fuelled by glycolysis are being oxidized into carbon dioxide, reducing equivalents and ATP. These reaction products are subse-

quently used for supporting pivotal cellular processes such as photosynthetic sucrose synthesis, nitrate reduction in the cytosol and hydroxypyruvate reduction in the peroxisomes, that will be described separately in this chapter. The high level of complicity and tight interconnection of these metabolic pathways located in various cellular compartments hamper our full understanding of the operation and regulation of mitochondrial respiration process. Surprisingly, even fundamental questions such as whether the cycle operates at all in the illuminated photosynthetic tissue (Padmasree, et al., 2002) remain unsolved. It is known that the functioning of the TCA cycle in the light is affected by a combination of several factors. First of all, the initial enzyme of the cycle, namely mitochondrial pyruvate dehydrogenase undergoes reversible inactivation in the light (Randall, et al., 1990). This multienzyme complex irreversibly converts pyruvate into acetyl-CoA and is though to be a key point regulating flux through the cycle. Secondly, the activity of the TCA cycle is influenced by the rapid export of its products outside mitochondria (Hanning and Heldt, 1993; Atkin, et al., 2000), for example in order to support glutamate synthesis (Hodges, 2002). The export of TCA cycle intermediates was shown to generate a significant flux *in vivo,* in specific developmental stages (Schwender, et al., 2003; Schwender, et al., 2006). Interestingly, recent studies performed on broad bean led to the conclusion of almost complete (95%) inactivation of the TCA cycle activity in the light (Tcherkez, et al., 2005). Similarly, microarray experiments showed a clear trend of reduced expression in the light of genes associated with respiratory processes (Blaesing, et al., 2005; Urbanczyk-Wochniak and Fernie, 2005; Urbanczyk-Wochniak, et al., 2006), although this does not necessary indicate limitated flux through the pathway. Nevertheless, the mitochondrial electron transport chain remains active irrespectively of illumination (Atkin, et al., 2000; Padmasree, et al., 2002; Yoshida, et al., 2006). This is feasible due to broad metabolic flexibility of the plant respiratory chain based on the existence of a cyanide-resistant non-phosphorylating pathway, a rotenone-insensitive oxidation site and the ability to oxidise external NAD(P)H (Hourton (Millenaar and Lambers, 2003; Rasmusson, et al., 2004; Vercesi, et al., 2006; Hourton-Cabassa and Moreau, 2008). Similarly, the TCA cycle possess a number of bypass reactions, including operation of a malic enzyme which sustains the functioning of TCA cycle, independently of glycolysis. The influence of the mitochondrial respiration on the selected intercellular processes is described in detail below. Detailed information on the mitochondrial role in the illuminated leaves can be found in recently published review (Nunes-Nesi, et al., 2008).

1.2.2.1 Provision of ATP for photosynthetic purposes

The photosynthetic ability is strongly connected to the functionality of respiratory pathways in plants. It is believed that one of the major functions of plant mitochondria is the production of ATP to support cytosolic sucrose synthesis. This theory is supported by the massive export of ATP from mitochondrial matrix into cytosol via highly active ATP:ADP translocator (Heldt, 1969). Moreover, the application of specific inhibitors revealed that the cytosolic ATP/ADP ratio de-

creased much more dramatically following the impairment of mitochondrial ATP synthase by oligomycin (Kromer and Heldt, 1991) rather than in conditions introducing limitations in photorespiration and oxidative phosphorylation (Gardestrom and Wigge, 1988). Subsequent experiments confirmed that the inhibition of oxidative phosphorylation results in a decrease of both activity and activation state of sucrose phosphate synthase (SPS) (Kromer, et al., 1993). It could be explained by the fact that the cytosolic sucrose synthesis is highly dependent on the supply of UTP, which is supplied by the conversion of mitochondrially produced ATP catalyzed by cytosolic nucleoside-5`-diphosphate kinase (NDK) (Kromer, 1995). In contrary to these finding, the analysis of wild type and a starch-less mutant of *Nicotiana sylvestris* suggested that the production of ATP in the mitochondria is not prerequisite for the maintenance of high rates of photosynthetic sucrose synthesis (Hanson, 1992). More recent experiments have showed that the function of respiration in photosynthetic metabolism is to both improve sucrose metabolism and modulate the flow of metabolites related to redox status (Igamberdiev, 1998; Igamberdiev, et al., 1998; Padmasree and Raghavendra, 1999; Padmasree, et al., 2002; Dutilleul, et al., 2005; Scheibe, et al., 2005). Further experimentation is required to clarify the correlation between these two vital cellular processes in plants, however to date, strong evidence has accumulated confirming that alterations in photosynthetic performance can be caused by modifications in activities of the selected TCA cycle enzymes (Carrari, et al., 2003a; Nunes-Nesi, et al., 2005b; Nunes-Nesi, et al., 2007a) and mitochondrial electron transport chain (Dutilleul, et al., 2003a; Bartoli, et al., 2005).

1.2.2.2 Generation of reducing equivalents to support photorespiration

The oxygenase activity of Rubisco produces phosphoglycolate under atmospheric conditions. In order to prevent wasteful loss of carbon, phosphoglycolate is converted to phosphoglycerate within the photorespiratory pathway, that reenters 75% of this carbon back into metabolism. This pathway converts two molecules of glycine into one molecule of serine with the simultaneous evolution of carbon dioxide, ammonium and production of NADH (Figure 5). The reactions are spatially separated and spread across chloroplast, mitochondria, peroxisome (Raghavendra, et al., 1998; Padmasree, et al., 2002; Bykova, et al., 2005) and, as recently found also cytosol (Timm, et al., 2008). The pathway has been studied intensively since the presentation of glycine decarboxylase mutant (Somerville and Ogren, 1983) and revealed coordinated expression and regulation of genes involved in mitochondrial photorespiration and Calvin cycle in Arabidopsis (Srinivasan and Oliver, 1995; McClung, et al., 2000). The reduction of hydroxypyruvate in the peroxisomal matrix requires the delivery of redox equivalents. Due to low cytosolic NADH concentration, only 1% of photorespiratory flux could be supported from this source (Reumann, et al., 1994). It is therefore believed that in the illuminated leaf under photorespiratory conditions the peroxisomal demand for reductants are met by internal oxidation of malate. This metabolite is simultaneously allocated to the peroxisomes from chloroplasts and mitochondria, by the use of malate-OAA

shuttle (Kroemer and Scheibe, 1996). More recent analysis of *ucp1* mutant, deficient in the expression of the uncoupling protein AtUCP1 allowed the elucidation of a mechanism that facilitates the oxidation of photorespiratory NADH in the mitochondrion (Sweetlove, et al., 2006). The uncoupling proteins (UCPs) are located within the inner mitochondrial membrane and they are responsible for dissipation of the proton gradient, which is normally used for ATP synthesis. The *ucp1* plants were characterized by reduced rates of both photosynthetic carbon assimilation and photorespiratory glycine oxidation. This finding stays in agreement with previously assigned role for the uncoupling protein in dissipating mitochondrial proton gradient as heat (Krauss, et al., 2005), which may influence the flux through TCA cycle (Smith, et al., 2004a). These data confirmed that the mitochondrial respiratory processes play an important role in the coordination of metabolism in the illuminated leaf (Kromer, et al., 1988; Raghavendra, et al., 1994; Carrari, et al., 2003a; Raghavendra and Padmasree, 2003; Bartoli, et al., 2005; Nunes-Nesi, et al., 2005a; Scheibe, et al., 2005).

1.2.2.3 Production and export of metabolites to sustain nitrate assimilation in cytosol

The assimilation of inorganic nitrogen into plant metabolism requires a reduction of nitrate in the cytosol. This process must be supported by a provision of carbon skeletons and reducing equivalents, which source remains a topic of debate. In general, this role appears to be performed by the TCA cycle (Fieuw, et al., 1995; Scheible, et al., 1997a; Scheible, et al., 1997b; Stitt, 1999; Stitt and Scheible, 1999; Masclaux, et al., 2000), although not all experimental observations support this theory (Galvez, et al., 1996; Kruse, et al., 1998; Lancien, et al., 1999). The cooperation between carbon and nitrogen metabolism in higher plants is described in more detail in the next section of this chapter.

1.3 Nitrogen metabolism in plants

1.3.1 The role of nitrogen in plant biology

Nitrogen is one of the most abundant elements in plants and it is quantitatively the most important for plant growth. It is a central precursor of many biological compounds, including: amino acids, purines and pyrimidines which are the building proteins and nucleic acids. Although nitrogen is present in the biosphere in a large variety of forms, the most abundant molecular nitrogen (N_2) is only accessible to a limited number of plants via symbiotic nitrogen fixation with bacteria (e.g. legumes). Higher plants assimilate easily inorganic nitrogen present in the soil in the form of nitrate and ammonium, in addition to uptake of organic N sources, like amino acids, which are abundant in soils that contain high concentrations of organic matter. Although nitrate is more abundant in the majority of soil types, due to the predominance of nitrifying bacteria (Marschner and Marschner, 1995), most plants take up ammonium preferentially, even if nitrate concentration is ten

times higher than that of ammonium (Crawford and Forde, 2002). Taking into account that N availability is a vital factor limiting plant growth and development and that world's most important crops are unable to fix nitrogen in symbiosis, the micronutrient uptake is strengthened in agriculture by massive use of fertilizers which number is predicted to extend over 200 million tons in 2050 (Galloway, et al., 2008).

1.3.2 Transport and assimilation of nitrogen

Plants have evolved numerous nitrate uptake systems to cope with variable nitrate levels in the soil. At the physiological level they are divided into two distinct groups: low affinity transport systems (LATS), which operate at high external NO_3 concentrations (above 1mM) and high affinity transport systems (HATS), which operate in a micromolar range. The LATS are low capacity, saturable systems, whilst HATS are high capacity systems, with linear, non-saturable uptake kinetics, as reported in number of recent reviews covering all aspects of nitrate uptake in plants (Crawford and Forde, 2002; Glass, et al., 2002; Orsel, et al., 2002; Orsel, et al., 2006; Miller, et al., 2007; Tsay, et al., 2007; Chen, et al., 2008; Camanes, et al., 2009). Both systems transport nitrate together with a proton (H^+) in a symport mechanism that is driven by the pH gradients across membranes. Two families of nitrate transporter genes encoding the LATS and HATS systems have been cloned from plants and named NRT1 and NRT2, respectively. NRT1 proteins belong to the oligopeptide transporters family (PTR super-family), while NRT2 proteins belong to the nitrate – nitrite porters (NNP super-family). Up to date fifty three NRT1 and seven NRT2 family transporters were identified in the Arabidopsis genome and their function and expression have been extensively studied in plant mutants (see the reviews listed above). Interestingly, similar LATS and HATS transport systems are responsible for uptake of ammonium and its distribution within plant (for reviews see von Wiren, et al., 2000a; von Wiren, et al., 2000b; Crawford and Forde, 2002; Glass, et al., 2002; Ludewig, et al., 2007; Ludewlg, et al., 2007; Miller, et al., 2008). The HATS are coded by six ammonium transporter (AMT) genes in *A. thaliana*, which subcellular localization, preferential tissue expression and knockout effects have been already studied. Apart from ions, organic nitrogen compounds can also be taken up from soil by roots and utilized for long distance N transport. Multiple plant transporters, shuffling amino acids, ureides and peptides, in addition to other N-containing metabolites were found and functionally characterized in model and crop plants, including Arabidopsis, tomato, potato, broad and castor bean, pea, barley and rice (for recent review see: Liu and Bush, 2006; Stacey, et al., 2006; Waterworth and Bray, 2006; Rentsch, et al., 2007). Although the roles of many of these transporters remain unclear, some may supply plants with N from soils that contain high concentrations of organic matter (Grossman and Takahashi, 2001).

Following the uptake of nitrate from soil via specific transporters in the plasmalemma, the ion is metabolized in the cytoplasm of root cells or stored and the

excess of nitrate is effectively exerted from roots (Figure 2). Alternatively, nitrate is being transported passively through xylem to the shoots where it is further metabolized or stored in leaf vacuoles to sustain general osmoticum and support growth in N limiting conditions (Taiz and Zeiger, 2002). Whether the reduction of nitrate takes place in roots or in leaves depends on the species and nitrogen conditions (Faure, et al., 2001). Once assimilated, nitrate is first reduced to nitrite by cytosolic nitrate reductase (NR), followed by further reduction to ammonium by chloroplastic nitrite reductase (NIR). Ammonium is subsequently incorporated into glutamine (Gln) and glutamate (Glu) via glutamine synthetase (GS) - glutamate synthase (GOGAT) cycle, which operates in cytoplasm and plastids of roots and shoots (Figure 2).

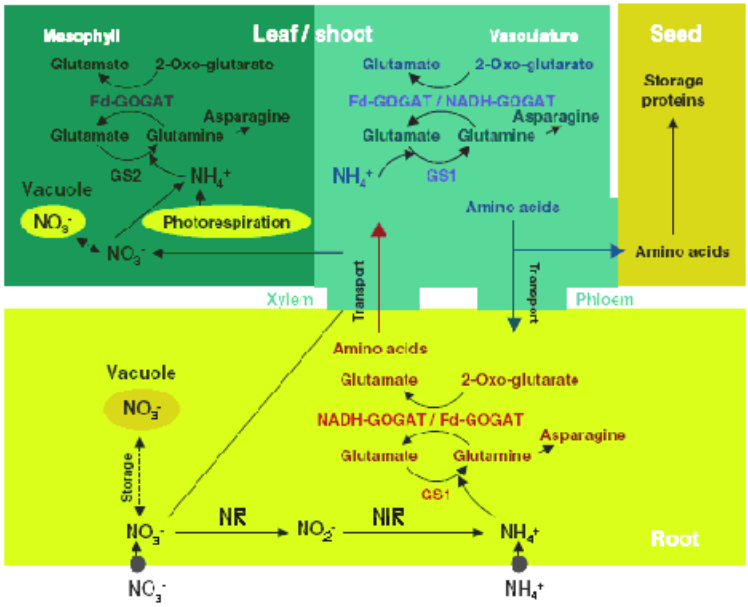


Figure 2: The schematic representation of the nitrogen assimilation and transport pathway in plants (after Suzuki and Knaff, 2005, slightly modified). The nitrate ions imported from soil and stored in vacuole are reduced to nitrite and subsequently into ammonium in roots and leaves. The ammonia ions, including those imported from soil and issued from different pathways are assimilated into glutamine (Gln) and glutamate (Glu) by the sequential reaction of glutamine synthetase (cytosolic GS1 and chloroplastic GS2) and glutamate synthase (Fd-GOGAT and NADH-GOGAT). Gln and Glu are used for amino acid, nucleic acid and protein biosynthesis. Amino acids are in part mobilized into the xylem for transport to the shoots or into the phloem for transport to other organs.

The studies performed on Arabidopsis, pea, barley, tobacco and other mutants revealed the presence of multiple GS and GOGAT isoforms that possess various organ- and compartment-specific expression patterns, as reviewed by (Coruzzi, 2003) and Suzuki and Knaff (Suzuki and Knaff, 2005). Moreover, chloroplastic glutamate synthase was found in higher plants to occur in three forms (NADP-, NADPH- and Fd-GOGAT), depending on their electron donor specificity. The GS-GOGAT isoforms localized in various tissues are subjected to different regula-

tory factors and are therefore believed to play several biological roles in primary N assimilation, N redistribution and transport as well as photorespiration. An alternative pathway for the formation of glutamate directly from ammonium can be achieved via mitochondrial glutamate dehydrogenase (NADH-GDH) and chloroplastic NADPH-dependent isoform of GDH. These enzymes catalyze a reversible Glu to 2-OG conversion and their actual physiological function in higher plants remain unsolved (see above mentioned reviews). The generation of Glu and Gln is followed by a biosynthesis of aspartate (Asp) and asparagine (Asn) by aspartate aminotransferase (AAT) and asparagine synthetase (AS). The primary assimilation of ammonium results therefore in the generation of these four, most abundant in leaves and roots amino acids that are subsequently used to translocate organic nitrogen from source to sink tissues. They provide nitrogen required for the formation of other amino acids and they help to control the nitrogen status during growth and development of plants.

1.3.3 Regulation of nitrogen transport and metabolism

Nitrogen is an essential macronutrient that influences plant growth, architecture and development, root branching, seed production, flowering and senescence time (Scheible, et al., 1997c; Stitt and Krapp, 1999; Corbesier, et al., 2002; Crawford and Forde, 2002; Stitt, et al., 2002; Pellny, et al., 2008), therefore N metabolism has to be tightly coordinated. A significant amount of evidence has been accumulated to prove that the genes regulating nitrogen assimilation are controlled at the transcriptional level, mainly by light and downstream carbon and nitrogen metabolites, including ions. The analysis of Arabidopsis, tobacco and tomato plants growing in N rich conditions revealed repressed transcription of several genes involved in N assimilation by ammonium and amino acids (Scheible, et al., 1997a; Scheible, et al., 1997b; Wang, et al., 2000; Wang, et al., 2001; Wang, et al., 2003; Scheible, et al., 2004; Wang, et al., 2004), although the expression of these genes was de-repressed when nitrogen became limiting for plant growth. The efficient nitrate uptake, precisely regulated at the transcriptional level due to the existence of constitutive (c) and inducible (i) elements in both HATS and LATS systems, allows plants to tightly control the acquisition of N, depending on its transient availability, form and concentration (Daniel-Vedele, et al., 1998; Forde, 2000; Crawford and Forde, 2002; Glass, et al., 2002; Orsel, et al., 2002; Miller, et al., 2007). Similarly, the expression of ammonium transporters is also regulated by nitrogen, as confirmed by the induction of *AMT* gene in shoots and roots within hours after subjecting to N deprivation (Howitt and Udvardi, 2000; von Wiren, et al., 2000b; Glass, et al., 2002).

Additionally, the expression of most of the nitrate and ammonium transporters as well as genes involved in primary nitrogen assimilation, such as NR, GS, ASN, Fd-GOGAT and NADH-GOGAT is light / diurnally regulated, however it can be abolished by addition of external sugars (as reviewed by Stitt and Scheible, 1999; Stitt, et al., 2002; Coruzzi, 2003; Foyer, et al., 2003; Thum, et al., 2003; Suzuki

and Knaff, 2005; Forde and Lea, 2007; Thum, et al., 2008).The diurnal changes in the level of various transcripts, enzymatic activities and metabolite concentrations are orchestrated in order to meet the demand for N supply and tune N acquisition from soil with the capacity to assimilate nitrate. The experiments performed by Kaiser (Kaiser, et al., 1999) and Scheible (Scheible, et al., 1997b) and their co-workers revealed that light stimulates translation of the NR transcript and moreover, post-translationally activates NR protein and inhibits its degradation. It results in massive increase of N assimilation in the beginning of the light period and elevation of level of ammonium, Gln, glycine (Gly) and serine (Ser), which serve as a storage pool of reduced N (Scheible, et al., 2000; Matt, et al., 2001b). The N assimilation is gradually decreased during the day and aborted at night, due to post-translational inactivation of NR activity in the dark. In contrast, nitrate uptake is constant during the day and only slightly decreases at night, whereas ammonium acquisition remains always at the high level (Matt, et al., 2001a). The mobilization of accumulated products increases by the end of the day and at night, accordingly to changes in the transcript and activity level of GS.

Since nitrate assimilation requires synthesis of organic acids, such as 2-oxoglutarate (2-OG) that acts as an acceptor for ammonium in GS - mediated reaction, and malate which prevents alkalisation during nitrate assimilation, the performance of major players in C metabolism has to be likewise adjusted to the diurnal changes. Indeed, it was found that phosphoenolpyruvate carboxylase (PEPCase), cytosolic pyruvate kinase (PK), mitochondrial citrate synthase (mCS), and cytosolic NADP-dependent isocitrate dehydrogenase (NADP-ICDH) undergo similar diurnal alterations in mRNA level and are regulated by nitrate (Figure 3)(Scheible, et al., 1997a). Moreover, nitrate was shown to inhibit transcription of ADP-glucose pyrophosphorylase (AGPase), a key enzyme for the regulation of starch synthesis. These studies provided a proof for tight coordination between primary nitrogen assimilation and carbon metabolism. Interestingly, plants seem to possess an excessive ability to assimilate N, that is fully utilized exclusively within a specific time frame during day/night cycle. This phenomenon could potentially extend plant adaptability to unfavorable conditions. Indeed, tobacco mutants Nia30(145) possessing very low nitrate reductase activity (Scheible, et al., 1997a; Scheible, et al., 1997b; Scheible, et al., 2000) survive due to altered diurnal rhythm of expression and enzymatic activity of NR, NIR, PEPCase, PK, mCS and NADP-ICDH, that was distinct from tobacco wild type plants subjected to both N replete and nitrate-deficient conditions. The reciprocal activation of NR, NIR and PEPCase in the early part of the light period favors generation of malate for pH regulation, whereas coordinated increase of PK, mCS and NADP-ICDH transcript and activity by the end of the day and at night prioritizes biosynthesis of 2-OG for assimilation of accumulated N sources (Scheible, et al., 1997a; Scheible, et al., 1997b; Scheible, et al., 2000; Stitt, et al., 2002; Fritz, et al., 2006a). The Arabidopsis NR-null mutant described by Wang and co-workers (Wang, et al., 2004) provided a great system that enabled to distinguish between the signaling effect of nitrate itself from the effect of metabolites downstream of nitrate reduction and

assimilation. Through the comparison between the transcriptome of NR-null mutant and wild type plant, the authors identified hundreds of nitrate-regulated genes that are involved in N, C and sulphur (S) metabolism. Interestingly, the response of the majority of these genes was limited to the certain plant organs. The localized regulation of transcripts is receiving increasing attention of scientists due to the recent development of technical tools that enable cell-specific profiling (Gifford, et al., 2008).

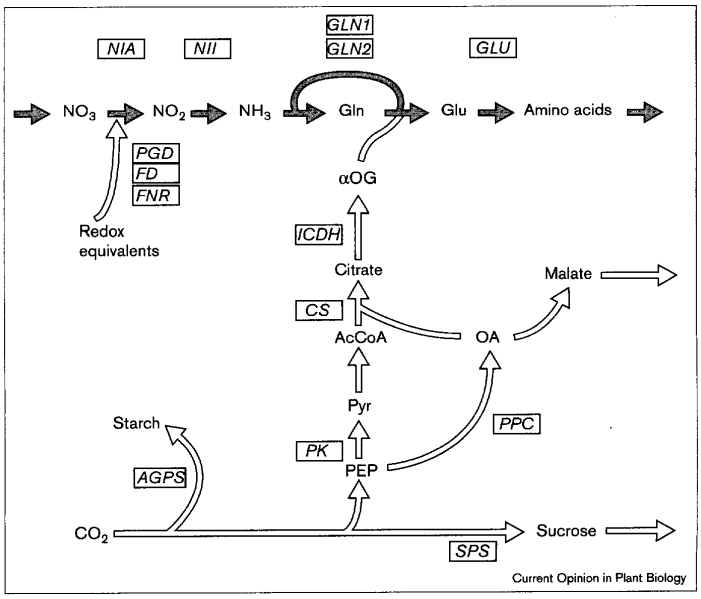


Figure 3: Interaction between C and N primary metabolism (after Stitt, 1999). The graph depicts primary nitrogen assimilation pathway (shaded arrows) and C metabolism pathways (open arrows) in plants. All genes, presented in boxes were found to be regulated by nitrate. Abbreviations: AGPS – ADP-glucose pyrophosphorylase (regulatory subunit), SPS- sucrose phosphate synthase, PK – cytosolic pyruvate kinase, PPC – phosphoenolpyruvate carboxylase, CS – citrate synthase, ICDH – NADP-isocitrate dehydrogenase, PGD – 6-phosphogluconate dehydrogenase, FD – ferredoxin, FNR – ferrodoxin:NADP oxidoreductase, NIA - nitrate reductase, NII– nitrite reductase, GLN1 - plastid glutamine synthetase, GLN2 – cytosolic glutamine synthase, GLU – glutamate synthase.

Although transcript levels of many genes show extensive diurnal variations in plants, the corresponding protein level does not always follow these changes. This is due to the existence of post-translational mechanism, which is essential for adaptation of metabolism to rapid changes in light intensity. The post-translational control could be achieved either by protein phosphorylation, which for example deactivates NR after a light-to-dark shift or by interactions with thioredoxins, as it was reported for all chloroplastic enzymes of primary nitrogen assimilation, that is NIR, GS and GOGAT (see Lemaitre, et al., 2007: for review). The experimental evidence accumulates that NR, GS and Fd-GOGAT are light-induced in parallel with genes involved in photosynthesis (Tepperman, et al., 2004; Jonassen, et al., 2008). This is true not only for photosynthetically active tissues, but also for roots,

in which gene transcription seem to be regulated both by light and sugars transported from shoots (Lejay, et al., 2008). The actual photosynthesis-dependent signal has still not been identified, although a review on light-enhanced N metabolism provides an excellent summary of recent knowledge of signaling cascades (Lillo, 2008).

Apart from post-translational control of N metabolism, plant response to altered nutrient supply is also regulated at the transcriptional and post-transcriptional level. To date, multiple transcription factors (TFs) involved in regulation of N metabolism have been found, as reviewed by Vidal and Gutierrez (Vidal and Gutierrez, 2008). The initial discovery of ANR1, a root-specific MADS-box protein that influenced lateral root production in Arabidopsis (Zhang and Forde, 1998) was followed by identification and functional characterization of TF members of GATA and DOF families (Yanagisawa, 2004; Bi, et al., 2005). Whilst the latter two TF families seem to have widespread effect on plant metabolism and work as integrators of C and N metabolism, the members of recently described myb-family of TFs provided a mechanistic link for integrating N nutrition and circadian regulation of gene expression in Arabidopsis (Gutierrez, et al., 2008). Recently, a number of microRNAs influencing the expression of genes regulated by nitrate and/or sucrose were discovered by microarray studies (Gutierrez, et al., 2007; Gifford, et al., 2008). The evidence for post-translational regulation was also found in Arabidopsis *nla* (nitrogen limitation adaptation) mutant (Peng, et al., 2007a; Peng, et al., 2007b). These plants revealed early senescence phenotype in low inorganic nitrogen conditions, due to disruption of RING-type ubiquitin ligase, which regulates plant adaptability to N starvation.

1.3.4 Nitrogen sensing and signaling

1.3.4.1 Markers of nitrogen status in plants

The assimilation of nitrogen requires provision of carbon skeletons and reducing equivalents. Signals providing information on internal and external N status are controlling expression of the genes involved in both N and C metabolism. One of such potent stimuli is nitrate, that has been proposed as a biochemical signal to influence plant growth, regulate lateral roots development and shoot:root ratio (Stitt and Scheible, 1999; Forde, 2002b: and 2002a; Scheible, et al., 2004; Zhang, et al., 2007; Vidal and Gutierrez, 2008). Nitrate induces a large number of genes, including those encoding high (NRT2) and low (NRT1) affinity nitrate transporters, NR, NIR, GDH, AS and the enzymes of GS-GOGAT pathway (see Stitt, 1999: for review; Wang, et al., 2000; Stitt, et al., 2002). This is an example of a well known mechanism of forward activation of a downstream pathway signaled by increased substrate availability. Moreover, nitrate regulates the expression of genes involved in different aspects of carbon metabolism, spanning from glycolytic pathway and the synthesis of organic acids used for both amino acid synthesis and regulation of pH in response to nitrate uptake and assimilation, up to the

oxidative pentose phosphate pathway, which provides high levels of NADPH needed for nitrate assimilation (Stitt, 1999; Wang, et al., 2001). The scientific tools developed in the post genomic era allowed the discovery of over thousand genes that respond to micromolar levels of nitrate within minutes (Gutierrez, et al., 2007). The existing homeostasis of cytosolic nitrate concentration in plants can be destroyed by alterations in environmental conditions and that fact confirms the theory of nitrate as a cellular signal of N status (as reviewed by Miller and Smith, 2008).

Recent studies concentrated also on the signaling effect of another N containing ion - nitrite, which physiological role is only starting to be disclosed (Stoimenova, et al., 2003; Igamberdiev, et al., 2005; Libourel, et al., 2006; Stoimenova, et al., 2007; Wang, et al., 2007). Nitrite is potentially a powerful signaling molecule, as its internal content has to be tightly regulated due to high toxicity for plant tissues. As revealed by early studies of NIR-deficient tobacco (Vaucheret, et al., 1992) and barley (Duncanson, et al., 1993) mutants growing on nitrate, accumulation of nitrite leads to severe growth retardations, chlorosis or even plant death. The analysis performed on nitrogen-starved *Arabidopsis thaliana* roots revealed over hundred of specifically nitrite–regulated genes (Wang, et al., 2007). Additionally, these studies pinpointed a large group of genes involved in the metabolism of carbon, nitrogen, sulfur, ammonium, energy and pentose phosphate pathway, that responded accordingly to both nitrate and nitrite short term treatment. Interestingly, high external concentration of ammonium suppressed the induction of several genes by nitrate and had varied effects on the nitrite response. Ammonium is presumably another signaling agent in plants, as it has been shown to affect the expression of several hundred genes in Arabidopsis roots (Fizames, et al., 2004). On the other hand, multiple members of central carbon metabolism are known to respond to alterations in internal and external N level and are believed to play a signaling role in plants.

A handful of major amino acids and organic acids play a pivotal role in this interaction between C and N metabolisms and some of them were proposed to serve as a markers of specific metabolic processes (see Lea and Forde, 1994; Lam, et al., 1998; Stitt, et al., 2002; Foyer, et al., 2003: for review). Foyer and co-workers (Foyer, et al., 2003) suggested Gln, Gln/Glu, Gln/2-OG, malate/2-OG and Asn as a useful set of metabolic markers for primary N assimilation, although it should be noted that these metabolites reflect also changes in photorespiratory rates and availability of ammonia and 2-OG (Ferrario-Mery, et al., 2002a; Foyer and Noctor, 2002; Novitskaya, et al., 2002; Foyer, et al., 2003). Nevertheless, multiple studies of tobacco NR knockout mutants and overexpressors confirmed that fluctuations in Gln content are significantly correlated with the extractable NR activity (Foyer, et al., 1994; Scheible, et al., 1997b). However, this association was broken in tobacco leaves supplied with 2-OG (Muller, et al., 2001). Although Gln is known to repress the NR transcript, it is the relative concentration of both Gln and

2-OG that exerts the control over NR transcription, as revealed by analysis of Arabidopsis mutants deficient in Fd-GOGAT activity (Dzuibany, et al., 1998; Ferrario-Mery, et al., 2001; Ferrario-Mery, et al., 2002b) and sulfur-deprived tobacco wild type plants (Migge, et al., 2000). Recent review on regulation of N acquisition confirms that among all exogenously applied amino acids, Gln is the most powerful in inhibiting both nitrate and ammonium influx and transporter transcript levels (Miller, et al., 2007). Moreover, cysteine (Cys) and asparagine (Asn) were also proposed to influence the regulation of N metabolism (Stitt, et al., 2002). The former amino acid stands at the crossroad between N and S metabolism and may tune N assimilation in response to changes in S availability. On the other hand, Asn signaling is combined with C source availability. Interestingly, the Asn level increases in low sugar supply conditions (Lam, et al., 1998), which is a diametrically different response than all other amino acids. It is believed that feedback inhibition by asparagine inhibits nitrate reduction in carbohydrate depletion environment (Stitt, et al., 2002). Later research confirmed involvement of Asn and Gln in N signal transduction pathway (Seebauer, et al., 2004).

A vital role within plant N signaling and regulation is assigned to glutamate (Glu). It is an immediate product of *de novo* N assimilation and N recycling during photorespiration (Coschigano, et al., 1998; Stitt and Krapp, 1999; Novitskaya, et al., 2002) and substrate for biosynthesis of many amino acids. Surprisingly, the internal level of Glu remains relatively constant during the diurnal cycle (Fritz, et al., 2006b) and in the large variety of nutrient deficient conditions tested on various plant species (Masclaux-Daubresse, et al., 2002; Matt, et al., 2002; Noctor, et al., 2002; Novitskaya, et al., 2002; Urbanczyk-Wochniak, et al., 2005; Fritz, et al., 2006a; Fritz, et al., 2006b). The stability of the Glu pool indicates that its concentration is tightly regulated (Hodges, 2002) and presumably, even small changes of the Glu level can trigger a feedback inhibition of nitrate assimilation (Fritz, et al., 2006a). Recently, it was proposed that Glu acts within a much broader network of N signaling pathways that enable the plant to monitor and adapt to alterations in N status and supply (for details see excellent reviews on signaling and Glu metabolism by both Lam, et al., 2006; Forde and Lea, 2007). An exceptional changes in Glu content occurred in the leaves of transgenic tobacco plants exhibiting decreased activity of Rubisco (RBCS) (Quick, 1994; Matt, et al., 2002; Fritz, et al., 2006a). They were however associated with alterations in 2-oxoglutarate (2-OG) content, similarly like in *N. sylvestris* CMS mutant (Dutilleul, et al., 2003a; Dutilleul, et al., 2005) which was disrupted in mitochondrial complex I. In the leaves of both of these transgenic plants alterations in the redox state inhibited the synthesis of 2OG, resulting in a decrease of Glu and increase of Gln. 2-OG is the major organic acid located at the interface between C and N metabolisms and essential for Glu synthesis (see brilliant review on the importance of 2-OG in plant metabolism in Hodges, 2002). 2-OG is generated mainly by various isoforms of isocitrate dehydrogenase enzymes and it serves as a direct provider of carbon skeletons for assimilation of nitrogen performed by GS-GOGAT cycle. The biosynthesis of 2-OG is therefore regulated by nitrate (Scheible, et al., 1997a; Scheible, et al.,

2000), changes in C metabolism and redox state. The precise physiological role of 2-OG in plants has not been fully discovered yet, however it is believed that this organic acid may be a sensor of leaf nitrogen status (Scheible, et al., 2000; Glass, et al., 2002; Hodges, 2002; Stitt, et al., 2002; Foyer, et al., 2003) and a presumable target of PII signaling proteins, as described in more detail in the next section of this thesis.

1.3.4.2 Nitrogen signaling cascades in prokaryotes

Nitrogen sensing and signaling has been an intensively studied, especially in prokaryotes and lower eukaryotes, since the discovery of the first N-regulatory (NTR) protein, PII (GlnB) in *E. coli* (Pahel, et al., 1978). The bacterial PII signal transduction protein senses the changes in key C and N metabolite and energy levels and coordinates the cell's response (Magasanik, 2000; Ninfa and Atkinson, 2000; Arcondeguy, et al., 2001; Moorhead and Smith, 2003; Jiang and Ninfa, 2007). PII does not have an enzymatic activity, but through the binding to the molecules of ATP, 2-OG and Gln it gathers information of the intracellular energy, C and N status, respectively (Figure 4). This information is subsequently used to regulate N metabolism by either promoting the transcription of the N-sensitive genes or by blocking the activity of GS enzyme. The first scenario takes place in high N conditions and is mediated by protein-protein interactions between PII and the N response regulator (NRII) of a two-component regulatory system. NRII activates the transcription factor NRI and thereby promotes the transcription of genes involved in N assimilation. The interaction between PII and NRII is however conditioned by a prior binding of 2-OG and ATP to PII, in order to ensure that high C and energy status were achieved before N assimilation is launched. When N availability decreases PII binds to ATPase that initiates posttranscriptional inactivation of GS by adenylation. The control over PII proteins is taken over by UT/UR, a uridylyltransferase and uridylyl cleavage enzyme, which modifies the performance of PII protein by adding or removing UMP. In the majority of prokaryotes, including plant-symbiotic rhizobia, the UT/UR activity is performed by GlnD enzyme, that regulates binding of PII protein to the AmtB ammonium transporter. This signal cascade construct a main bacterial pathway for ammonium assimilation. According to current state of knowledge, the GlnD enzyme is considered the most important sensor of bacterial nitrogen status, as reviewed by (Gruswitz, et al., 2007; Leigh and Dodsworth, 2007; Yurgel and Kahn, 2008). Recent results indicate that GlnD is important for nitrogen-fixing symbiosis between rhizobia and legume plants, as mutation in this gene resulted in ineffective support of alfalfa growth (Yurgel and Kahn, 2008).

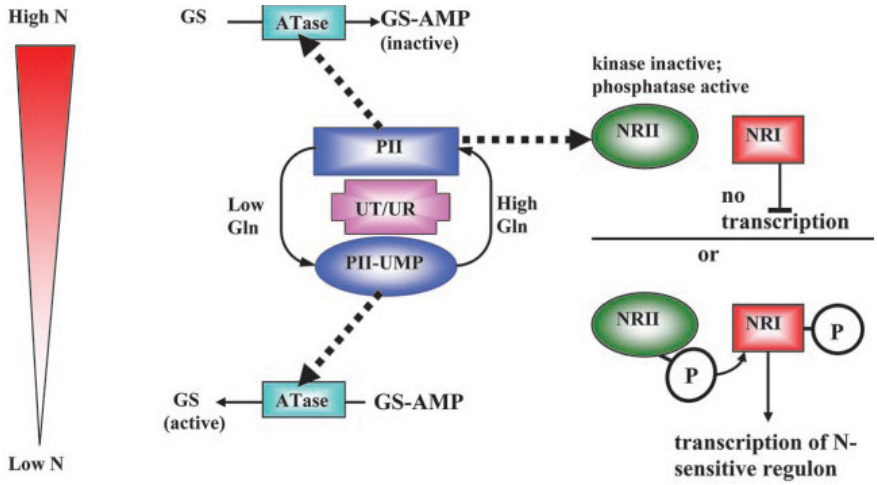


Figure 4: Model of bacterial NTR regulatory system (after Moorhead and Smith, 2003). During conditions of N sufficiency, cellular levels of Gln promote deuridylylation of PII and subsequent interaction of unmodified PII with ATase, which adenylates GS to inactivate the enzyme. In parallel, PII binds N regulator II (NRII) to suppress its protein kinase activity and activate its phosphatase activity, maintaining the transcription factor NRI in a dephosphorylated, inactive state. When Gln levels drop and both the C and energy status is interpreted as adequate, PII is uridylylated by the UT/UR. PII-UMP now stimulates the activation of GS and, in the absence of affinity for NRII, allows NRII to activate NRI and promote transcription of the N-sensitive regulon. Thick dashed lines represents protein:protein interactions. Substrates for the covalent modification reactions (UTP and ATP) are not shown for simplicity.

In *Escherichia coli*, exist a distinct from NTR signaling system that allows for sensing of very low concentrations of extracellular respiratory oxidants. It is a dual two-component regulatory system NarX-NarL and NarQ-NarP that regulates anaerobic respiratory-gene expression in bacteria. The sensors NarX and NarQ are consisting of histidine protein kinases (HPKs), capable of autophosphorylation in response to nitrate and nitrate. They control the phosphorylation state of response regulator NarL and NarP that possess the ability to bind target operons, such as electron transport components and anaerobiosis-specific nitrate reductase (Chiang, et al., 1997; Stewart, 2003; Jiang and Ninfa, 2007; Noriega, et al., 2008).

1.3.4.3 Nitrogen sensing and signaling in higher plants

Very little in known about nitrogen signaling in plants, although there are homologues of prokaryotic regulatory proteins in plant genome. First plant PII gene (GLB1) was identified in *Arabidopsis thaliana* and castor bean (Hsieh, et al., 1998), however to date single PII homologues were found also in tomato, rice, aflafla, pine and algae (Hsieh, et al., 1998; Sugiyama, et al., 2004; Mizuno, et al., 2007a; Mizuno, et al., 2007b). Plant PII is a nuclear-encoded chloroplastic protein that shares 50% similarity to bacterial proteins. The transcription of plant *GLB1* was shown to be induced by light and sucrose and repressed by addition of specific amino acids, such as Asn, Gln, or Glu (Hsieh, et al., 1998). The Arabidopsis

plants constitutively overexpressing PII gene suffered from impaired ability to sense or metabolize Gln, which supported the theory of plant PII acting as a putative C/N sensor, similarly to bacterial homologues. The sequence and structure analysis of plant GLB1 revealed that Arabidopsis PII is a homotrimer that binds with high affinity to small molecules, such as: ATP, ADP and 2-OG, however it is unable to covalently attach Gln or other amino acids (Smith, et al., 2003). Further investigation confirmed that plant PII is not regulated by phosphorylation or uridylylation in response to nitrogen metabolites, in contrast to the bacterial proteins (Smith, et al., 2004b). Nevertheless, the crystal structure of Arabidopsis PII (Mizuno, et al., 2007a) resembles the bacterial trimer with three protruding T-loops on one surface, although it possesses also a plant-specific N-terminal extension on the opposing surface, that may be involved in protein-protein interactions and signaling. Very recent studies of PII-NAGK complexes in cyanobacteria (Llacer, et al., 2007) and Arabidopsis (Mizuno, et al., 2007b) revealed highly similar crystals containing MgATP bound to PII and NAG, ADP and arginine bound to NAGK. The N-acetyl-L-glutamate kinase (NAGK), which is a key enzyme in the biosynthesis of arginine, was shown to interact with cyanobacterial and plant PII protein, as revealed by yeast two-hybrid studies performed on Arabidopsis and rice (Burillo, et al., 2004; Sugiyama, et al., 2004). The physiological role of higher plant PII protein in the regulation of arginine biosynthesis was confirmed by discovering the 50% decrease of arginine, ornithine and citrulline levels in PII T-DNA insertion mutants grown under ammonium nutrition conditions (Ferrario-Mery, et al., 2006). Arginine is an ideal N storage metabolite, as it is easily mobilized N–rich amino acids. Its biosynthesis is feedback inhibited at the NAGK level (Slocum, 2005). When N deficiency occurs, 2OG accumulated due to low GS-GOGAT activity, binds PII together with ATP and therefore promotes dissociation of PII-NAGK complex and activates NAGK (Llacer, et al., 2007). Interestingly, the PII Arabidopsis mutants were very sensitive to nitrite toxicity and had increased light-dependent nitrite uptake into chloroplasts (Ferrario-Mery, et al., 2008). These results confirm the role of higher plant PII in regulation of nitrite acquisition and translocation, reminiscent of its function in cyanobacteria (Lillo, 2008).

Regardless from PII signal transduction system, an important role in N sensing status may play glutamate receptors, analogous to mammalian ionotropic iGluRs receptors responsible for nerve signal transmission in synapses (Forde and Lea, 2007; Miller, et al., 2007). To date, 20 genes encoding putative glutamate receptor genes (*AtGLR*s) were identified in Arabidopsis (Chiu, et al., 2002), however only one (AtGLR1.1) seems to be directly involved in regulation of C and N metabolism. The Arabidopsis antisense AtGLR1.1 seeds were unable to germinate in the presence of sucrose, unless co-supplied with nitrate (Kang and Turano, 2003). These plants were further characterized by a significant decrease in the activity and transcript level of key C- and N-metabolic isoenzymes. The authors proposed that AtGLR1.1 functions as a sensor and regulator of C and N status and that the metabolic response to variable conditions is mediated by abscisic acid (ABA).

Another group of plant hormones, namely cytokinins play presumably a central role in signalling plant N status (Inoue, et al., 2001). Not only the concentration of active cytokinins in plants changes concomitantly with nitrogen supply (Takei, et al., 2002), but also the enzymes of cytokinins biosynthetic pathway were shown to be specifically activated upon nitrate treatment (Miyawaki, et al., 2004).

1.4 Crosstalk between carbon, nitrogen and energy metabolism in plants

A close interaction between C and N metabolism exist in higher plants (Figure 5). Both photosynthesis and carbohydrate breakdown provide C skeletons, reducing equivalents and ATP for assimilation of inorganic N and biosynthesis of amino acids and nucleotides (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Foyer, et al., 2003; Escobar, et al., 2006). On the other hand, N-containing metabolites are required to allow C to be utilized for growth. Multiple genes involved in primary C and N metabolism are regulated by the same signaling molecules, such as for example nitrate (Figure 3). Moreover, genes involved in energy metabolism, especially in reduced ferredoxin production, and in NAD(P)H synthesis by the pentose phosphate pathway were also shown to be nitrate-induced (reviewed by Redinbaugh and Campbell, 1991; Stitt, 1999; Wang, et al., 2000). The correlation between C, N and energy metabolism is very well visualized in cytoplasmic male sterile II (CMS) mutant. The *N. sylvestris* plants lacking mitochondrial complex I of the respiratory electron transport chain (Gutierres, et al., 1997) were characterized by increased respiration, in addition to decreased photosynthetic efficiency, modified light acclimation responses and enhanced stress resistance (Sabar, et al., 2000; Dutilleul, et al., 2003a; Dutilleul, et al., 2003b; Dutilleul, et al., 2005; Priault, et al., 2006a; Priault, et al., 2006b). These plants revealed altered internal C to N balance, as predicted on the basis of vast changes in leaf metabolite content when grown in N replete conditions and increased shoot/root ratio at low N (Dutilleul, et al., 2005). Recent work on tobacco plants elucidated some of the regulatory mechanisms combining N supply with changes in respiration performance and plant architecture, through hormone signaling (Pellny, et al., 2008).

The actual source of C skeletons required for N assimilation by GS-GOGAT cycle remains a topic of debate. Such role is frequently assigned to mitochondria, since citrate is known to be a main metabolite exported from these organelles (Hanning and Heldt, 1993), the enzymes capable of conversion of citrate to 2-OG are localized in cytosol (Chen and Gadal, 1990; Galvez, et al., 1999; Carrari, et al., 2003a; Abiko, et al., 2005a; Abiko, et al., 2005b) and there is an efficient mechanism for plastidial 2- OG uptake (Renne, et al., 2003). The demand for cytosolic 2-OG supply could be met either by cytosolic or mitochondrial isocitrate dehydrogenases, or by aspartate aminotransferases. The direct evidence for such function of the cytosolic aspartate transferase was provided by studies of Arabidopsis AAT2 mutant, exhibiting growth retardation and dramatic light-dependent reductions in Asp and Asn level (Schultz, et al., 1998). However, although numerous experimental data sets provided valuable information concerning the actual physiological role

of cytosolic NADP-ICDH and mitochondrial NAD-IDH in 2-OG provision for N assimilation, they remain largely inconsistent, which renders any conclusions impossible to date. The detailed literature review on this topic is presented in the IDH chapter of this thesis.

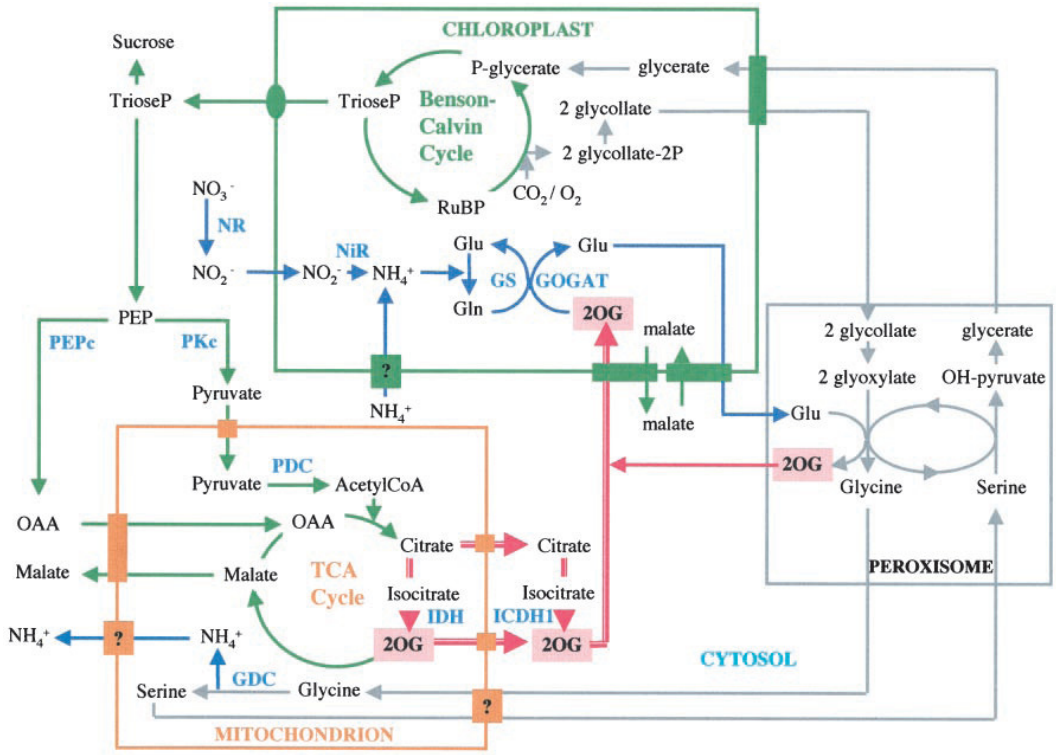


Figure 5: A simplified scheme showing C and N flow between organelles in plant leaves. The possible involvement of isocitrate dehydrogenases in 2-OG production for ammonium assimilation is shown (after Lancien, et al., 2000). In higher plant leaves, ammonium formed via GDC during photorespiration in mitochondria or formed from nitrate by NR and NiR activity is assimilated in the chloroplasts by the action of the GS/GOGAT pathway. Two pathways involving isocitrate dehydrogenases have been proposed to supply 2-OG for this purpose. In one pathway 2-OG, synthesized in the mitochondria by the NAD-IDH is exported to the cytosol via a 2-OG transporter. In the second pathway, citrate is exported from the mitochondria to the cytosol and metabolized to 2-OG by the sequential action of an aconitase and the cytosolic NADP-ICDH (ICDH1). In both cases, 2-OG is transported from the cytosol to the chloroplast by a 2OG/malate translocator. Photorespiratory ammonium re-assimilation by the chloroplastic GS/GOGAT cycle is coupled to peroxisome metabolism via the cycling of Glu and 2-OG between these two organelles. This metabolite shuttling is controlled by a double translocator system composed of chloroplastic 2-OG and Glu translocators. A malate-oxaloacetate (OAA) shuttle is important in the maintenance of low mitochondrial NADH levels and the transfer of reducing equivalents to the peroxisome for glycerate synthesis from hydroxy-pyruvate (OH-pyruvate) during photorespiration. See the text for abbreviations; RuBP-ribulose 1,5-bisphosphate.

1.5 General aim of the thesis

The broad aim of this work was to perform a functional characterization of selected TCA cycle enzymes in plants. Specifically, I concentrated on mitochondrial citrate synthase (mCS), and both cytosolic (NADP-ICDH) and mitochondrially (NAD-IDH) located isocitrate dehydrogenases. In order to reach the aim of this work, several strategies were applied. Firstly, screening of the tomato EST collection for the selected TCA cycle genes. Secondly, generation of the antisense and RNAi constructs and their subsequent introduction into tomato plants, aiming at the disruption of the endogenous genes of interest. Thirdly, screening of obtained transgenic plants, followed by full characterization of the selected lines at the physiological, metabolic and transcript levels in the photosynthetically active tissue (leaves) in carbon and nitrogen-replete conditions. Additionally, investigation of phenotypic and metabolic alterations of NADP-ICDH and NAD-IDH plants subjected to N-deficiency stress was performed. Summarizing, this work was designed to provide further information concerning the function and regulation of TCA cycle in the illuminated leaf of tomato. In the broader context, the obtained experimental results may help to elucidate some gaps in current understanding of co-ordination of C, N and energy metabolism in higher plants.

2 MATERIALS AND METHODS

2.1 Commonly used equipment, kits and consumables

All chemicals, kits and enzymes used in this study were obtained from Roche Diagnostics (Mannheim, Germany) or Invitrogen (Karlsruhe, Germany) with the exception of radiolabelled sodium bicarbonate and D- (1-^{14}C) and D- (6-^{14}C) glucoses which were from Amersham International (Braunschweig, Germany), D- (2-^{14}C) and D- (3,4-^{14}C) glucoses were from American Radiolabeled Chemicals (ARC, St. Louis, MO, USA), and ^{13}C pyruvate which was from CIL (Cambridge Isotope Laboratories, Inc, Andover, MA, USA).

2.2 Transformation and cultivation of bacteria

For transformation purposes I used *Escherichia coli* strains Xl-1 Blue Stratagene, La Jolla, CA, USA (Bullock, et al., 1987), whereas *Agrobacterium tumefaciens* strains used were GV2260 (Deblaere *et al.,* 1985) and GV3101 (Koncz and Schell, 1986). Chemically competent *E.coli* cells were transformed by heat-shock as described by Hanahan (1983). After transformation, cells were grown at 37°C on LB medium supplemented by selective antibiotic as described by Sambrook (1989). Competent *A.tumefaciens* cells were prepared according to Höfgen and Willmitzer (1990) and transformed by electroporation according to Miller (1988). The cells were grown at 28°C on YEB medium supplemented with selective antibiotic according to Vervliet (1975). Bacterial glycerol stocks were generated as described by Sambrook (1989) and stored at -80°C for further use.

2.3 Tomato DNA source

Full length cDNAs encoding for CS and IDH/ICDH genes were isolated following screening of the tomato EST collection of Clemson University Genomics Centre (http://www.genome.clemson.edu). The database, described by van der Hoeven and collaborators (Van der Hoeven, et al., 2002) is publicly available and was generated with support of the National Science Foundation Plant Genome Program (http://www.tigr.org/tdb/tgi/lgi; http://www.sgn.cornell.edu). At the beginning of the project the database revealed four tomato TCs annotated to mitochondrial and peroxisomal CS and twelve TCs annotated to various NAD-IDH and NADP-ICDH isoforms, however, due to incomplete tomato genome sequencing process, only five of them possessed partial but higher than 60% sequence similarity to respective TCA genes from other organisms. Initial screening of bacteria containing the EST clones annotated to encode for CS and IDH/ICDH genes were inoculated in LB liquid medium supplemented with 100 mg.L^{-1} ampicilin. The resultant cultures were screened by PCR, which contained *Taq* DNA polymerase 10X buffer (Invitrogen, USA), 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 0.30 μM of each Lac Z specific primers (forward LacZ1

5'GCTTCCGGCTCGTATGTTGTGTG3' and reverse LacZ2 5'AAAGGGGGATGTGCTGCAAGGCG3') and Taq DNA polymerase (Invitrogen, USA). The amplified fragments were run on agarose gels and sequenced (SeqLab, Germany). For confirmation of the fragment size plasmid DNA from EST clones was extracted using the NucleoSpin kit (Macherey-Nagel GmbH, Germany) and digested by restriction enzymes (Roche Diagnostics, Germany). The criterion of selection of the putative full length clones was the homology with the orthologous genes from *Arabidopsis thaliana* and other available species.

2.4 DNA manipulation

DNA manipulations were performed essentially as described by Sambrook (1989). Plasmid DNA was extracted using the NucleoSpin Exctraction kit (Macherey-Nagel GmbH, Germany) and PCR mix was purified using NucleoSpin Purification kit (Macherey-Nagel GmbH, Germany). For transcript profiling purposes, first- and second-strand cDNA synthesis mix were cleaned up by Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and concentrated using a Microcon YM-30 column (Millipore).

2.5 Plant material and standard growth conditions

Solanum lycopersicum cv. Moneymaker was obtained from Meyer Beck (Berlin, Germany). Plants were handled as described by Carrari and co-workers (2003a). Tomato seeds were surface-sterilized (ethanol 70% 2 min; 6 % Nahypochlorite + 0.1 % triton X-100 solution, 20 min; wash 3 times in sterile water) and plated onto AMOZ media (0.24 % Murashige and Skoog-Medium; 0.055 % MES) supplemented with 2% (w/v) sucrose, 50 $\mu g.ml^{-1}$ kanamycin for selection and 0.7% agar. Plates were kept in growth chamber (250 µmol photons m^{-2} s^{-1}, 22°C under a 16-h/8-h day light) until it was possible to differentiate kanamycin resistant seedlings. Following the transfer of seedlings to a vermiculite-soil mixture, they were kept in the greenhouse under following conditions: minimum of 250 µmol photons m^{-2} s^{-1} light intensity, 22°C under a 16-h/8-h day light regime.

2.6 Hydroponic growth conditions during nitrogen starvation stress

Plants (*Solanum lycopersicum* cv. Moneymaker) subjected to nitrogen limitation experiment were germinated from seeds on AMOZ medium (0.24 % Murashige and Skoog-Medium; 0.055 % MES) containing 2% (w/v) sucrose and 0.7% agar and were grown in a growth chamber under a light intensity of 500 µmol photons m^{-2} s^{-1}, at 25 °C under a 12/12 h light/dark regime for one week. Subsequently, seedlings were transferred to hydroponic boxes (40x30x11.3 cm Alliber, Muehlheim, Germany) containing complete tomato nutrient solution ($Ca(NO_3)_2$ 1.25 mol m^{-3}, KNO_3 1.5 mol m^{-3}, $MgSO_4$ 0.75 mol m^{-3}, K_2HPO_4 0.83 mol m^{-3}, FeEDTA 0.05 mol m^{-3}, H_3BO_3 11.6 mmol m^{-3}, $MnSO_4$ 2.4 mmol m^{-3}, $ZnSO_4$ 0.2 mmol m^{-3}, $CuSO_4$ 0.1 mmol m^{-3}, $NaMoO_4$ 0.1 mmol m^{-3}) (Baumeister and Ernst, 1978)

under a 12/12 h light/dark regime at 25°C, constant (50%) relative humidity and high light conditions (900 µmol photons m^{-2} s^{-1}). After one week, plants were subjected to either nitrogen replete [4 mM NO_3^-] or deficient [0.4 mM NO_3^-] conditions. To minimize the effect of the altered nitrogen content on osmotic potential when nitrate was reduced to 0.4 mM, the nutrient solution was augmented by an equimolar mixture of calcium sulphate and potassium sulphate to maintain the same cation concentration as that of the replete nutrient medium. Samples were taken solely from PCR-confirmed transgenic and wild type leaves after twenty days of growth in stable nitrogen conditions.

2.7 Cloning of selected tomato cDNAs into destination vectors

Sequencing of purchased mitochondrial CS tomato clones, all belonging to the TC155277 tentative consensus revealed full length 1568 bp gene encoding an open reading frame of 471 amino acids. For cloning purposes, I ligated the 1195bp fragment of mCS gene in the antisense orientation into pBinAR (Liu, et al., 1999), between the cauliflower mosaic virus 35S (CaMV) promoter and octopine synthase (*ocs*) terminator (Figure 6). While the 734 bp fragment of mitochondrial NAD-IDH tomato gene *Sl*IDH1 (TC193092) was cloned in the antisense orientation into pK2WG7 Gateway destination vector (http://www.psb.ugent.be/gateway/index.php?NAME=pK2GW7&_app=vector&_act=construct_show&), the 527 bp fragment of NADP-ICDH tomato gene (TC202045) was introduced into pK7GWIWG2(I) RNAi Gateway vector (http://www.psb.ugent.be/gateway/index.php?NAME=pK7GWIWG2(I)&_app=vector&_act=construct_show&) in both antisense and sense orientation that were split by a 643 bp intron. In both Gateway vectors the gene of interest was inserted between 35S promoter and T35S terminator (Karimi, et al., 2004). Both the NADP-ICDH cloning construct and NADP-ICDH transgenic tomato plants were generated in Max-Planck Institute for Molecular Plant Physiology in Potsdam-Golm, Germany and were provided to me due to the kindness of Dr Alisdair Fernie. The cloning was performed by the use of Gateway technology (Invitrogen, Karlsruhe, Germany) (Figure 7) according to manufacturer advices, using self-designed, attB-site containing primers (Table 1) and BP clonase enzyme mix (Invitrogen, Karlsruhe, Germany). Following heat-shock transformation of chemically competent *E.coli* and extraction of bacterial plasmid DNA, the presence and correct orientation of the insert were verified by antibiotic resistance, PCR and restriction-digestion analysis. Positive entry clones were generated by utilizing the pENTR Directional TOPO cloning kit for the Gateway system and subsequently subjected to LR recombination reaction (Invitrogen, Karlsruhe, Germany) in order to transfer gene of interest into expression vectors. Final destination clones, selected after successful *E.coli* transformation were partially sequenced (SeqLab, Germany) and introduced into competent *A.tumefaciens* cells for further transformation of tomato leaf tissue.

Table 1: Primers used for cloning of selected DNA fragments into entry vector pENTR by Gateway technology. This table presents the sequences of specific primers used for amplification of mCS, *Sl*IDH1and *Sl*ICDH1 tomato genes for cloning purposes. The capital letters represent attB1 and attB2 cloning sites in forward and reverse primers, respectively.

Primer type	Sequence of primer (5' to 3')
mCS forward	GGGG ACA AGT TTG TAC AAA AAA GCA GGC T at atg gtt ctt ggt gga atg
mCS reverse	GGGG AC CAC TTT GTA CAA AGC TGG GT a tgc ttt ctt gca ctg gtt c
*Sl*IDH1 forward	GGGG ACA AGT TTG TAC AAA AAA GCA GGC T gc acg agg agg aaa tgt agg
*Sl*IDH1 reverse	GGGG AC CAC TTT GTA CAA AGC TGG GT t gac aaa aat gat aca ata aat ga
*Sl*ICDH1 forward	GGGG ACA AGT TTG TAC AAA AAA GCA GGC T tt gat gac atc gat ccc tga
*Sl*ICDH1 reverse	GGGG AC CAC TTT GTA CAA AGC TGG GT c cag ggt taa gtg tgg att agt g

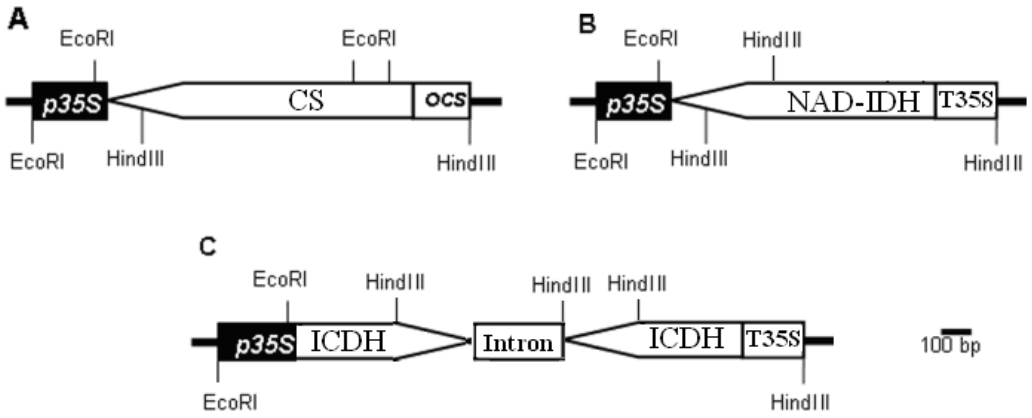


Figure 6: Final generated constructs introduced to tomato plants. Graphs A and B represent vectors pBinAR and pK2WG7carrying selected fragments of mCS gene (TC155277) and *Sl*IDH1 (TC193092) in antisense orientation, respectively. Graph C shows pK7GWIWG2(I) vector ligated with two fragments of *Sl*ICDH1 (TC202045) gene disrupted by an intron. Additionally, the figure presents enzyme restriction sites used for confirmation of correct size and position of inserts during cloning. Abbreviations: p35S-promoter , OCS, T35S – terminators.

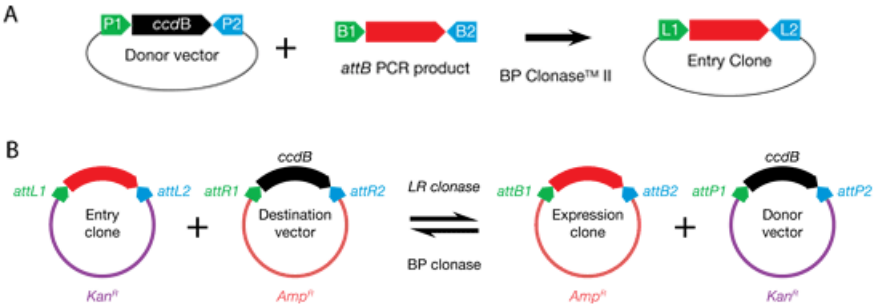


Figure 7: Schematic representation of Gateway cloning strategy. A. The upper line represents generation of entry clone by introducing attB-flanked PCR product into donor vector due to BP clonase enzyme mix activity. B. The lower line shows *in vitro* recombination between entry clone (pENTR) and destination vector (pBinAR, pK2WG7 and pK7GWIWG2(I)) that generates final expression clone ready for further use.

2.8 Phylogenetic analysis tomato CS and IDH/ICDH genes

Protein sequences were retrieved from the GenBank through the BLASTp algorithm using tomato mCS gene (TC155277), *Sl*IDH1 (TC193092) and *Sl*ICDH1 (TC202045) as query. With the aim of establishing copy number only sequences from eukaryotes and prokaryotes with fully sequenced genomes were selected. The tBLASTn algorithm was also used in order to search for non-annotated proteins. Sequences were aligned using the ClustalW software package (Higgins and Sharp, 1988: ; www.ebi.ac.uk/clustalw) using default parameters. Neighbor Joining trees (Saitou and Nei, 1987) were constructed with MEGA4.0 software (Tamura, et al., 2007). Distances were calculated using pair-wise deletion and Poisson correction for multiple hits, bootstrap values were obtained with 500 pseudo replicates.

2.9 RNA extraction and transcript quantification

Total RNA was isolated using the commercially available Trizol kit (Gibco BRL, Karsruhe, Germany) according to the manufacturer´s suggestions for the extraction from plant material. The microarray cDNA hybridization was carried out using the ESTs for the mitochondrial isoforms of citrate synthase (TC155277) and NAD-IDH (TC193092) obtained from the Clemson State University collection. Due to their relatively low expression, the transcript levels of all other isoforms of CS and IDH were determined by an established qPCR method (Czechowski, et al., 2004), using primers based on the ESTs of the Clemson State University collection (Table 2). Quality control of the synthesized double-stranded cDNA was performed by real time PCR approach using the tomato specific primers for GAPDH and ubiquitine (Table 2). Microarray analysis was carried out by the use of glass slides containing arrayed tomato ESTs (TOM1) obtained directly from The Center of Gene Expression Profiling (CGEP) at the Boyce Thompson Institute (BTI), Cornell University, The Geneva Agricultural Experiment Station, the

USDA Federal Plant and Nutrition Laboratory, exactly as described in Urbanczyk-Wochniak *et al* (2006). Signal intensities of four independent hybridizations of wild type and transgenic cDNA pools were quantified using GeneSpotter software version 2.3 (MicroDiscovery, Berlin, Germany) and analyzed and normalized using the special statistical software package R (R Development Core Team, 2004). Final data were visualized using MAPMAN software (Thimm, et al., 2004; Usadel, et al., 2005).

Table 2: Primers involved in transcript profiling of transgenic and wild type plants. The table presents sequences of tomato specific primers self-designed in order to analyze transcript level of all publicly available sequences of tomato CS and IDH/ICDH isoforms and their homologues. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitine specific primers were use to determine quality and quantity of cDNA obtained after reverse transcription of total RNA or messenger RNA. All sequences are presented in 5' to 3' orientation. Additionally, the table provides information concerning melting temperature of each primer and the length of expected amplicon, when used in pairs.

Annotation	TC	Forward primer	Reverse primer	Tm (°C)	Amplicon lenght (bp)
Mitochondrial CS	TC155277	TTGGATTGCCGCTAGAGAGG	TTGCACTGCTTCTCAAGCCA	80	61
Peroxisomal CSI	TC162670	TCCGAAGCAGACAAGCTCG	CAGCCAATCGTCGTTTGGT	84	61
Peroxisomal CSII	TC165584	CATCCTTGGAAAGGCACCTG	GTGTCTTCCAGCCATCCTCAA	81	61
Mitochondrial regulatory NAD-IDH (*Sl*IDH1)	TC193092	AGCGTGTTATTGCCGAAGGT	TGGGTGGTGCAGTCTCCA	79	61
Mitochondrial regulatory NAD-IDH (*Sl*IDH2)	TC198615	TTGTGGATAATTGCTGCATGC	CCCATACCATGACATCAAATGCT	78	61
Cytosolic NADP-ICDH (*Sl*ICDH1)	TC202045	CATTGCCTCCATCTTTGCCT	GTTGTTGTCCAATGTTGCCCT	82	61
Most likely cytosolic NADP-ICDH (*Sl*ICDH2)	TC164449	ACCAGCACAAATAGCATTGCC	TTGCCCTATGTGCAAGTCCA	79	61
Most likely mitochondrial NADP-ICDH (*Sl*ICDH3)	TC196623	GTGTTGCTTCCATTTTTGCATG	TTCCCATCAAGCTGAGCCTT	79	61
Ubiqitine	AK246708	GGTTAAGCTCGCTGTGTTGCA	CCTCCAGCCTTGTTGTAAACGT	80	61
GAPDH 3'	TC161943	TGTGTTTTAGTTTTTGCTTGGAAGTACT	CAAGAAAGACCACTCCAGGCC	76	61
GAPDH 5'	TC161943	AGCAATGGCAAACGGAAAGAT	CCAAACGACCAATTCTACCGAA	77	62

2.10 Analysis of enzyme activities

Enzyme extracts were prepared as described previously (Gibon, et al., 2004a), except that Triton X100 was used at a concentration of 1% and glycerol at 20%. CS, aconitase, NAD-IDH, ATP-phosphofructokinase, fumarase and pyruvate

kinase were assayed as described in Nunes-Nesi *et al* (2007a). Succinyl-CoA ligase was assayed as described in Studart-Guimaraes *et al*. (2005), whereas NADP-MDH was assayed as described in Scheibe and Stitt (1988). AGPase, phosphofructokinase (PPi), SPS, Cyt-FBPase, glucokinase, fructokinase, G6PDH, NADP-ICDH, shikimate dehydrogenase, GLDH, PEP carboxylase, acid invertase, Fd-GOGAT, NR, GS and NAD-GDH were assayed as described in Gibon *et al*(2004a). Cytosolic and plastidial PGI were assayed as described in Weeden and Gottlieb (1982). PGM was assayed as described in Manjunath *et al*. (1998), whereas glycerate kinase was quantified as detailed in Huege (2007).

2.11 Determination of metabolite and ion levels in tomato leaves

All metabolites were measured from leaf samples frozen in liquid nitrogen and stored at -80°C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer, as described by Nunes-Nesi *et al.* (2005b). All samples were harvested in the middle of the day, unless otherwise stated.

2.11.1 GC-MS – based metabolite profiling

The levels of several organic acids, amino acids, carbohydrates and secondary metabolites were analyzed by gas chromatography - mass spectrometry (GC-MS) approach exactly following the protocol described by Roessner *et al* (2001) with the exception that peak identification was optimized to tomato tissues (Roessner-Tunali, et al., 2003). The analysis was carried out using a GC/MS system consisting of an AS 2000 autosampler, a GC 8000 gas chromatograph and a Voyager quadrupole mass spectrometer (ThermoQuest, Manchester, GB). Prior to the injection into the system all samples were combined with the retention time standard mixture containing heptanoic, nonanoic, undecanoic, tridecanoic and pentadecanoic acid 3.7% (w/v) each, tricosanoic and nonadecanoic acid 7.4% (w/v) each, heptacosanoic acid 22.2% (w/v) and hentriacontanoic acid 55.5% (w/v) in tetrahydrofuran at 10 mg/ml total concentration. The chromatograms and mass spectra were evaluated using the Masslab program (ThermoQuest, Manchester, GB). A retention time and mass spectral library for automatic peak quantification of metabolite derivatives was implemented within the Masslab method format. For evaluation of relative values within each chromatogram the peak areas derived from specific ion traces indicative for each analyzed compound were normalized to the total peak area derived from all measured metabolites present within the same chromatogram. For reliable quantification at least six replicates were measured and standard errors (SE) were determined.

2.11.2 HPLC-based metabolite profiling

Quantification of α- and β-chlorophylls, β-carotene, lutein, neoxantin, violaxantin, antheraxanthin and zeaxantin level was performed by HPLC approach in 80%

acetone extracts as described in Bender-Machado *et al*. (2004). Tocopherols were measured from 100 mg of frozen leaf tissue according to the method described by Thompson and Hatina (1979). Nitrate levels were determined as detailed in Fritz *et al*. (2006b). Soluble amino acids were quantified in combined ethanol/water extracts from from fully developed leaves collected at six time points across a diurnal period. The extracts were stored at −20°C and subsequently subjected to the reverse phase high performance liquid chromatography (RP-HPLC) using an ODS column (Hypersil C_{18}; 150- × 4.6-mm i.d.; 3 µm; Knauer GmbH, Berlin) connected to an HPLC system (Dionex, Idstein, Germany). Amino acids were measured by precolumn derivatization with OPA in combination with fluorescence detection (Lindroth and Mopper, 1979), as described by Kreft *et al*. (2003). Peak areas were integrated by using Chromeleon 6.30 software (Dionex) and subjected to quantification by means of calibration curves made from standard mixtures.

2.11.3 Enzymatic assay-based metabolite profiling

The levels of starch, sucrose, fructose and glucose in the leaf tissue were determined photometrically exactly as described previously (Fernie, et al., 2001b). Samples were collected from fully developed leaves at six time points across a diurnal period. The change in absorbance was continuously detected at 340 nm using an Anthos ht II microtiter-plate reader (Anthos Labtec Instruments, Hanau, Germany). The quantification of soluble sugars was performed in the reaction mixture consisting of 20 µl ethanolic extract and 275 µl of 100 mM imidazol (pH 6.9), 5 mM $MgCl_2$, 2 mM $NADP^+$, 1 mM ATP and 2U ml^{-1} $NADP^+$-linked glucose-6-phosphate dehydrogenase (yeast). To start the reactions, the respective enzymes were sequentially added to the following final concentrations: for measurement of glucose content: 1 U ml^{-1} hexokinase (yeast overproducer), for fructose: 0.5 U ml^{-1} phosphoglucose isomerase (yeast), and for sucrose: 11.5 U ml^{-1} invertase (β-fructosidase from yeast). Starch content was measured using a commercially available starch determination kit (UV method; Cat.No. 207 748, Roche, Mannheim). The assay was based on the enzymatic hydrolysis of starch by α-amyloglucosidase and the determination of glucose in a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase. Nucleotides and nucleotide sugars were separated by HPLC on a Partisil-SAX anion-exchange column (P10SAX-250; Hichrom, Reading, UK) as described by Fernie at al. (2001b). Eluted nucleotides were detected by their A_{254} and identified and quantified by cochromatography with authentic nucleotide standards. The recoveries of small representative amounts of metabolites throughout the extraction, storage, and assay procedures have been documented previously (Fernie, et al., 2001a). The extraction and cycling assay of NAD, NADH, NADP and NAPH was performed according to the method described by Gibon and Larher (1997).

2.12 Measurements of photosynthetic parameters

Fluorescence emission was measured *in vivo* using a PAM (Pulse Amplitude Modulation) fluorometer (Walz, Effeltrich, Germany), prior to measurement of chlorophyll fluorescence yield and relative rate of electron transport (ETR). For this purpose, I used 4-6 week-old plants maintained at irradiances ranging from 0 to 1200 µmol photons m^{-2} s^{-1} for 30 min. The data were calculated using the Win-Control software package (Walz, Effeltrich, Germany). Gas-exchange measurements were performed in a special custom-designed open system (Lytovchenko, et al., 2002). The calculation of dark respiration and maximal photosynthetic efficiency was performed due to the use of a Licor-6400 gas-exchange system (Li-Cor; http://www.licor.com/) under 400 ppm CO_2 and leaf temperature of 25°C. The ^{14}C-labelling pattern of sucrose, starch, and other cellular constituents was performed by illuminating leaf discs (10 mm diameter) in a leaf-disc oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK) in saturating $^{14}CO_2$ at a PFD of 250 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation at 20 °C for 30 min and subsequent fractionation was performed exactly as detailed by Lytovchenko *et al.* (2002).

2.13 Measurement of respiratory parameters

Dark respiration was measured using the same gas exchange system as defined above. Estimations of the TCA cycle flux on the basis of $^{14}CO_2$ evolution were carried out following incubation of isolated leaf discs (6 mm diameter) in 10mM MES-KOH (pH 6.5) containing 2.32 KBq ml^{-1} of [1-^{14}C], [2-^{14}C], [3:4-^{14}C] or [6-^{14}C]glucose. $^{14}CO_2$ evolved was trapped in 10% KOH solution and quantified by liquid scintillation counting. The results were interpreted following ap Rees and Beevers (1960).

2.14 Measurement of redistribution of isotope

The fate of ^{13}C labelled pyruvate was traced following feeding of leaves excised from 6 week-old plants via the petiole placed in a solution containing 10mM MES-KOH (pH 6.5) and 20 mM [U-^{13}C]-pyruvate for three hours. The experiment was performed in a growth chamber under a light intensity of 500 µmol photons m^{-2} s^{-1}, at 25 °C. Fractional enrichment of metabolite pools was determined exactly as described previously (Roessner-Tunali, et al., 2004; Tieman, et al., 2006) and label redistribution was expressed as per Studart-Guimarães *et al.* (2007).

2.15 Statistical analysis

The *t* tests were performed using the algorithm embedded into Microsoft Excel (Microsoft, Redmond (WA)). The term significant is used in the text only when

the change in question has been confirmed to be significant ($P < 0.05$) with the t test.

3 Analysis of the function of mitochondrial citrate synthase on tomato leaf metabolism.

3.1 Introduction

The initial enzyme of the TCA cycle, citrate synthase (CS, E.C. 2.3.3.1) is believed to be a rate limiting step in the mitochondrial pathway, potentially controlling the flux through the cycle. It is responsible for the catalysis of the condensation reaction between the methyl carbon of acetyl-CoA and the keto-carbon of oxaloacetate (OAA), through the production of unstable citroyl-CoA, which is immediately hydrolysed to citrate. The final product of the reaction serves as a substrate for the aconitase protein which converts it into isocitrate, which itself is subsequently used by isocitrate dehydrogenase to generate 2-oxoglutarate (2-OG). 2-OG is likely a regulatory metabolite coordinating mitochondrial, cytosolic and chloroplastic metabolism, being involved in C-skeleton production for amino acid synthesis and cellular redox regulation (Scheible, et al., 2000; Glass, et al., 2002; Hodges, 2002; Stitt, et al., 2002; Foyer, et al., 2003). Due to the key position of mitochondrial CS in respiratory metabolism, much attention has been directed to investigate the performance of this enzyme. Since the elucidation of the amino acid sequence (Bloxham, et al., 1981), and crystallographic structure of CS from porcine heart (Remington, et al., 1982), the enzyme has been isolated from many prokaryotes and eukaryotes. The cDNA of mitochondrial CS isoform has been cloned from *Arabidopsis* (Unger, et al., 1989), potato (Landschutze, et al., 1995a; Landschutze, et al., 1995b), pummelo (Canel, et al., 1996), tobacco (LaCognata, et al., 1996), poplar tree (LaCognata, et al., 1996), sugar beet (LaCognata, et al., 1996), carrot (Takita, et al., 1999; Koyama, et al., 2000), various citrus fruits (Sadka, et al., 2001), tobacco (Delhaize, et al., 2001), strawberry (Iannetta, et al., 2004) and other species. These studies characterized citrate synthase mainly on the genetic, proteomic and enzymatic level, however detailed reports describing metabolic and physiological role of the enzyme in plants are still missing. Apart from animal science, only three studies concerning the inhibition of the enzymatic activity in higher eukaryotes were reported to date, namely research performed on *Solanum tuberosum, Podospora anserine* and *Saccharomyces cerevisiae.* The mCS antisense transgenic potato plants presented displayed flower formation and a high frequency of abortion deriving from the ovaries disintegration and leading to female sterility (Landschutze, et al., 1995b). Similarly, the sole observed effect resulting from the mCS gene inactivation in the filamentous fungus *P.anserine* mutant was the arrest of meiosis process at prophase (Ruprich-Robert, et al., 2002). In contrast to these findings, the presence of active mitochondrial isoform of CS in yeast was required for respiration and metabolism of acetate (Kispal, et al., 1988). Although yeast gCS failed to substitute mCS for TCA cycle sustenance, a single one of these isoforms was sufficient to prevent cells from glutamate auxothrophy (Lee, et al., 2006). More recent studies of *S. cerevisiae* have revealed that mCS mutants display glu-

tathione depletion and increased formation of reactive oxygen species (ROS) leading the researches to propose a protective role of the enzyme from heat- and age-induced apoptosis (Lee, et al., 2007).

The glyoxysomal isoform of citrate synthase has so far been cloned only from pumpkin (Katto, et al., 1995; Stevens, et al., 1997) and Arabidopsis (Pracharoenwattana, et al., 2005). The latter research revealed two active gCS genes expressed in plants, whose disruption led to altered seed dormancy and an inability to metabolize triacylglycerol (TAG). In contrast, yeast gCS mutants were able to use TAG for respiration due to activity of acylcarnitine shuttle, the presence of which remains controversial in plants (Wood, et al., 1992; Lawand, et al., 2002). The blockage of fatty acids beta-oxidation in the absence of glyoxysomal CS provides support for the importance of its metabolic function in plants, wherein it seems to be required for carbon export from the peroxisome, in the form of citrate.

Despite the paucity of research focussed on CS role in plant metabolism *per se* much research effort was directed at elucidating the role of citrate synthase in plant – soil interactions, especially in the field of phosphate uptake and aluminium (Al) tolerance of plants (de la Fuente, et al., 1997; Lopez-Bucio, et al., 2002). An increased enzymatic activity was proposed to associate with higher ability to accumulate citrate within plant tissues and greater efflux of this metabolite outside root cells. The independent studies of overexpression of mCS in Arabidopsis (Koyama, et al., 2000) and in tobacco (Lopez-Bucio, et al., 2000) revealed enhanced phosphorus scavenging ability from the soil. The higher citrate exudation from the root tissue of the latter transgenics improved ion availability in the soil and in consequence increased leaf and fruit yield under P-limiting conditions. Similarly, recent work on transgenic tobacco (Deng, et al., 2009) and canola plants (Anoop, et al., 2003) have shown that overexpression of mCS gene resulted in the enhanced tolerance to Al toxicity. Although up regulation of mCS in many plant species has increased Al resistance there has been no consensus regarding the effect of Al on internal organic acid content or CS activity. The majority of Al toxicity studies revealed either little increase in CS activity (Li, et al., 2000; Yang, et al., 2000) or unaltered enzyme performance in both Al resistant and Al sensitive varieties (Hayes and Ma, 2003; Zhao, et al., 2003; Liu, et al., 2007); Zhao *et al*., 2003). The effect of the TCA cycle activity on the biosynthesis of organic acids has also been studied in several important agronomic species, with the accumulation of some metabolites, especially citrate and malate being found to be highly regulated during fruit development and affect strongly fruit acidity and taste (Etienne, et al., 2002).

Depending on the precise physiological role of citrate synthase in different species, the regulatory mechanisms of the enzyme vary across living organisms. ATP feedback inhibition is evident in respiring animal cells, in which the major function of mCS is to produce energy in the form of ATP via oxidative phosphoryla-

tion. In anaerobic organisms, wherein this role is taken by glycolytic fermentation and the TCA cycle acts mainly as a donor of biosynthetic precursors and reducing power the mCS remains unaffected by ATP, however it is inhibited by NADH, an ultimate product of the pathway. In contrast, peroxisomally localized isoform of CS is independent of both cofactors (Dennis, 1997).

The regulation of mCS transcript and enzymatic activity differ depending on the plant species studied (Sadka, et al., 2001). The typical regulatory mechanism of a biosynthetic enzyme, i.e. massive induction on mRNA level, followed by an increase of activity and concentration of reaction product has previously been reported for mitochondrial CS in young leaves of potato (Landschutze, et al., 1995a), *Arabidopsis* (LaCognata, et al., 1996) and during lemon fruit development (Sadka, et al., 2001). Interestingly, an opposite tendency was found in potato flower anthers and pollen (Landschutze, et al., 1995b) as well as in strawberry flowers and fruits (Iannetta, et al., 2004). The inverse relationship between enzyme translation and activity suggests the presence of post-transcriptional regulation mechanism of mCS. Similar situations occur during seed germination and seedling growth, which may explain the lack of correspondence between the protein content and mRNA level of glyoxysomal CS in pumpkin (Katto, et al., 1995). In summary, the regulation of citrate synthase activity *in vivo* appears to be controlled at the transcriptional, translational and mRNA and protein stability levels (Sadka, et al., 2001) depending on various developmental and environmental stimuli (Iannetta, et al., 2004). That said the direct influence of modification of the activity of citrate synthase on metabolism of the illuminated leaf remains unknown.

3.2 Aim of work

The function of citrate synthase is undoubtedly bound to both TCA and glyoxylate cycle activity which provide organic acids and energy components. Although the activity of this enzyme has been postulated to influence all stages of plant life, the precise physiological role of CS in respiratory and photosynthetic performance of plants is as yet unclear. The aim of this chapter was to evaluate the importance of the mitochondrial citrate synthase within the metabolism of the illuminated leaf. For this purpose I generated transgenic tomato plants with decreased mitochondrial citrate synthase activity and completed a comprehensive characterization of them at biochemical, physiological and transcriptional levels.

3.3 Results

3.3.1 Sequence analysis and generation of transgenic CS plants

In order to functionally characterize the mitochondrial citrate synthase of tomato, antisense transgenic plants were generated. The gene was obtained following screening of a publicly available tomato expressed sequence tag (EST) collection

(Van der Hoeven, et al., 2002). The investigation of seventeen tomato ESTs, all belonging to a single tentative consensus (TC180568), revealed the structure of mCS gene composed an open reading frame of 470 amino acids and mitochondrial peptide sequence. Comparison with functionally characterized citrate synthases revealed 91% identity to *Solanum tuberosum* (Q43175), 85% to *Arabidopsis thaliana* CSY4 (At2g44350), 83% identity to *Arabidopsis thaliana* CSY5 (At3g60100), 56 % identity to *Saccharomyces cerevisiae* Cit1p (NP_014398), 54% Cit2p (NP_009931) and 45% identity to *Saccharomyces cerevisiae* Cit3p (NP_015325) which are all mitochondrial isoforms and less than 30% identity to peroxisomal isoforms (Figure 8). A transformation construct was generated by the introduction of a 1195bp fragment of the mCS ORF into the pBinAR vector under the control of 35S promoter. The *Agrobacterium* - mediated transformation of tomato leaves generated several positive transgenic plant lines. Total cellular citrate synthase activities were assessed, revealing several lines with decreased CS activity in comparison to wild type plants (Figure 9, Figure 10-left). Nine selected transformants were subsequently screened by the use of Northern blotting technique (Figure 10-right). Considering all that, four transgenic tomato lines displaying either strong (lines CS22 and CS40) or moderate (lines CS25 and CS45) reduction in CS expression were chosen for further investigation.

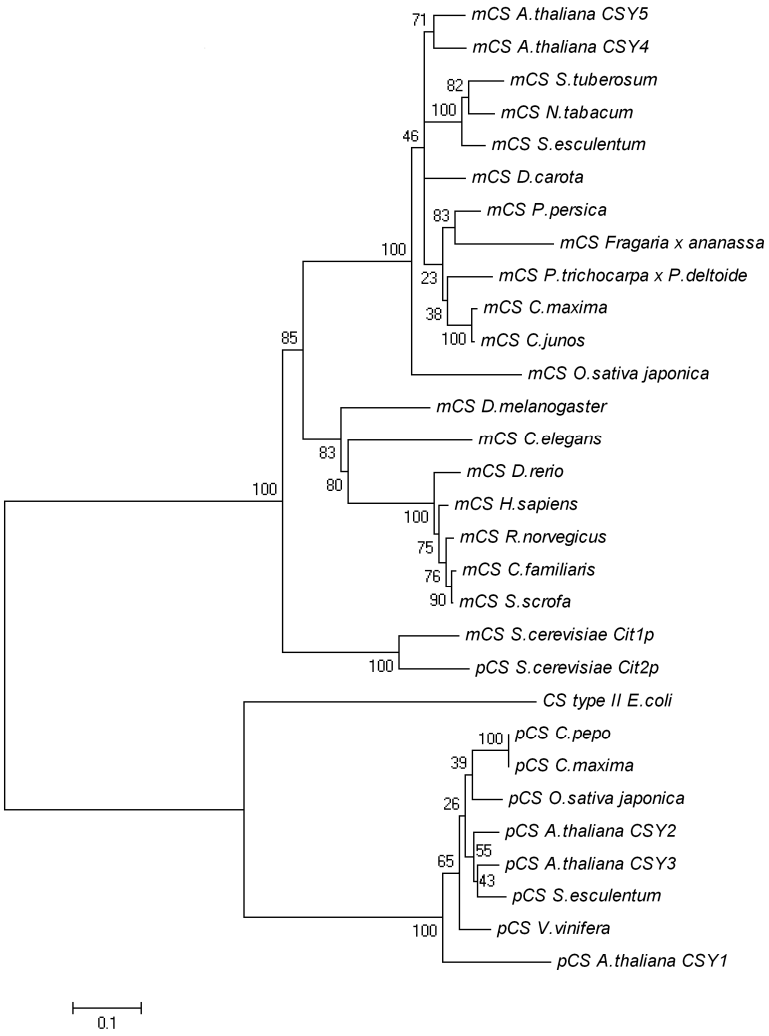


Figure 8: Dendogram of citrate synthase sequences. Citrate synthase sequences were aligned using the ClustalW alignment program (Higgins and Sharp, 1988). Neighbor Joining tree (Saitou and Nei, 1987) was constructed with MEGA4 software (Tamura, et al., 2007). The taxonomic names of the species and the accession numbers of sequences used in the phylogenetic analysis are: *mCS Solanum esculentum* (TC15527); mCS *Arabidopsis thaliana* (CSY4 NP_566016.1); mCS *Arabidopsis thaliana* CSY5 (NP_191569); mCS *Citrus junos* (AAR88248.1); mCS *Citrus maxima* (P49298); mCS *Daucus carota* (O80433); mCS *Fragaria x ananassa* (P83372); mCS *Nicotiana tabacum* (CAA59008.1); mCS *Oryza sativa japonica* (EAZ22071); mCS *Populus trichocarpa x Populus deltoides* (CAA59009); mCS *Prunus persica* (AAL11504); mCS *Solanum tuberosum* (Q43175); mCS *Vitis vinifera* (CAO67184.1); mCS *Bos taurus* (NP_001038186.1); mCS *Canis familiaris* (XP_531634.2); mCS *Caenorhabditis elegans* (NP_499264.1); mCS *Drosophila melanogaster* (NP_572319.2); mCS *Danio rerio* (XP_001337429.1); mCS *Homo sapiens* (AAC25560); mCS *Rattus norvegicus* (EDL84868); mCS *Sus scrofa* (NP_999441.1); CS type II *Escherichia coli* W3110 (BAA35384); mCS *Saccharomyces cerevisiae* Cit1p (NP_014398); pCS *Saccharomyces cerevisiae* Cit2p(NP_009931); pCS *Solanum esculentum* (TC174083); pCS *Arabidopsis thaliana* CSY1 (NP_191433.1); pCS *Arabidopsis thaliana* CSY2 (NP_191434.1); pCS *Arabidopsis thaliana* CSY3 (NP_181807.1); pCS *Cucurbita maxima* (P49299); pCS *Curcurbita pepo* (D38132); pCS *Oryza sativa japonica* (EAZ22338); pCS *Vitis vinifera* (CAO70044.1).

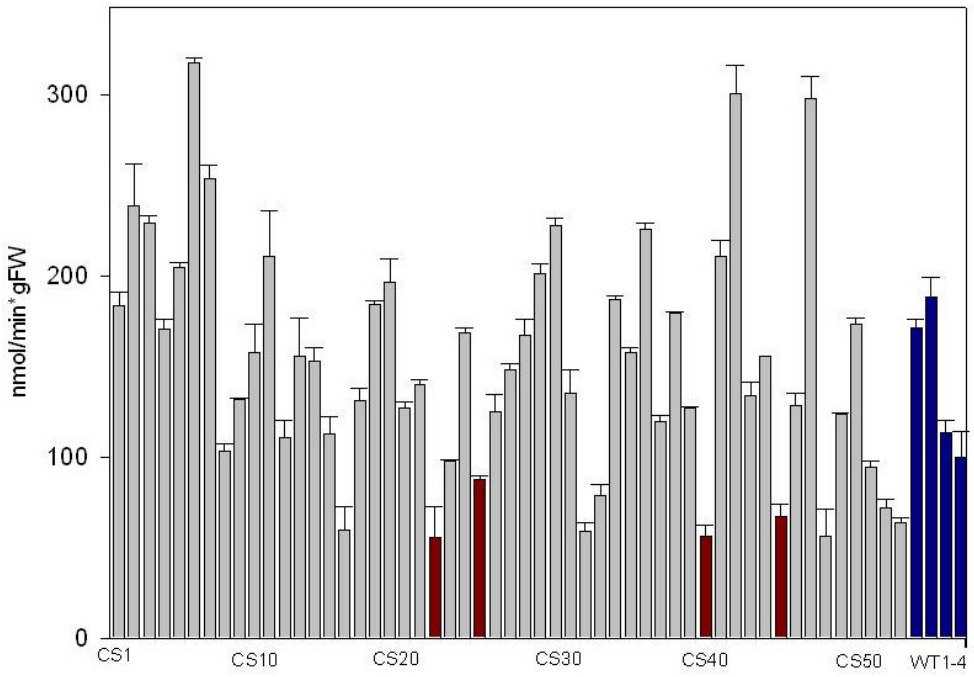


Figure 9: Total citrate synthase enzymatic activity in the leaves of all obtained transgenic lines (in gray) and wild type plants (in dark blue). The samples were collected from 6-week-old fully expanded source leaves at midday. The four CS lines selected for further investigation are presented in dark red. Values are presented as mean ± SE of three independent measurements on one plant per line.

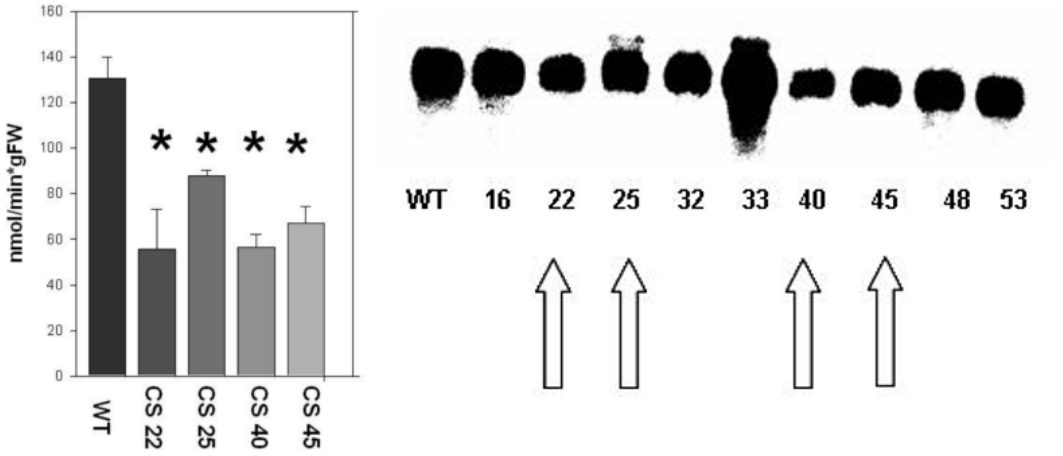


Figure 10: Enzymatic activity (on the left hand side) and transcript level (on the right hand side) of the citrate synthase in the 6-week-old source leaves of selected transgenic lines and wild type plants. The enzymatic assay shows total CS activity in leaves, whereas northern blot results presented on the on the right hand side graph are specifically limited to mRNA content of mitochondrial CS isoform due to the use of cDNA probe obtained from tomato mCS gene by PCR approach. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the Student's t test.

3.3.2 Phenotypic characterization of the transgenic CS plants

The selected transgenic plants were clonally propagated and grown side by side with wild type tomatoes in standard greenhouse conditions for ten weeks prior to phenotypical examination. Surprisingly, the CS transgenics exhibited no specific phenotype in comparison to the wild type plants (Figure 11). Both fresh and dry weights of investigated organs were unchanged in the transgenic plants however a slight tendency for increased dry weight of roots was noticeable (Figure 12). The strongest transgenic lines CS22 and CS40 also presented a mild, insignificant reduction of total dry weight of plants. Moreover, although the transgenics exhibited a trend toward decreased fruit yield, this was only significant in the case of line CS22 (data not shown). When the organ weight was presented as a percentage of total dry weight it was noticeable that the stem of transgenics was significantly greater than that of the wild type (data not shown), however that did not influenced total height of CS plants at any time point during development (Figure 13). Similarly, the number of branches and internodes remained comparable between genotypes (data not shown). Another interesting trait of the genetically engineered plants was their slightly lighter colour than the control plants. Subsequently, I turned my attention to floral tissues since antisense inhibition of the potato homolog of SlmCS has previously been characterized to confer a specific floral phenotype (Landschutze, et al., 1995b). Surprisingly, the insight into plant flowering did not reveal any significant changes between compared genotypes, showing approximate flowering time and only a slight trend of decreased number of flowers across the whole development period (Figure 14).

Figure 11: Photograph of five week old CS transgenic and wild type plants growing in the greenhouse conditions.

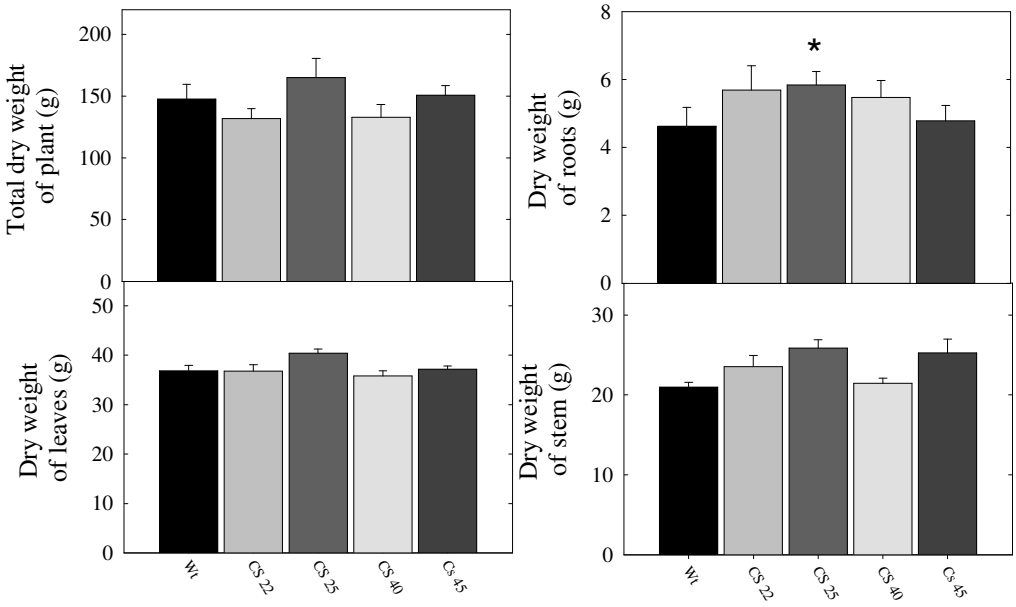


Figure 12: Dry weight of different plant organs and whole eleven week old CS transgenic and wild type plants growing in greenhouse conditions. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the Student's t test.

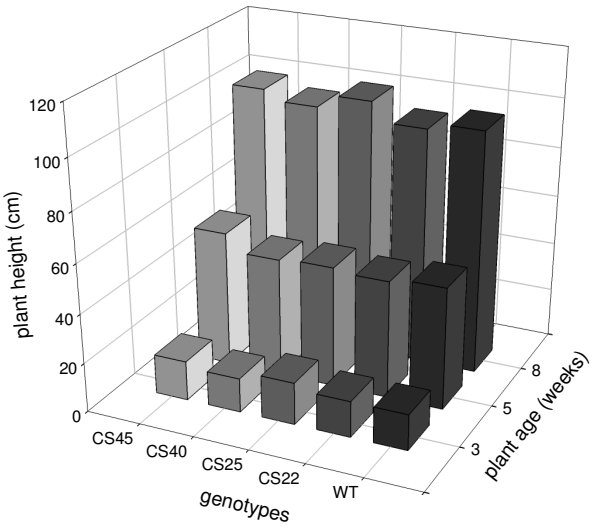


Figure 13: The comparable rate of growth of CS transgenic and wild type tomatoes in three measurements across plant development in greenhouse conditions. The values represent mean of six individual plants per line.

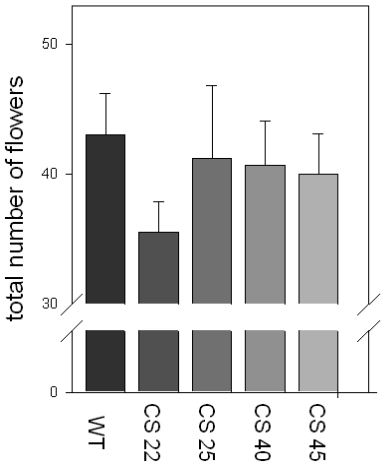


Figure 14: Total number of flowers generated by CS transgenic and wild type plants growing in greenhouse conditions until senescence. Values are presented as mean ± SE of determination on six individual plants per line.

3.3.3 Evaluation of the photosynthetic and respiratory parameters of the transgenic CS plants

Having noticed that down-regulation of the mitochondrial citrate synthase activity had little effect on the plant appearance, I next decided to investigate consequences of the transformation at the physiological level. For this purpose two approaches were adopted, namely radiolabelled carbon feeding experiments and gas exchange analysis.

The first approach was based on the measurement of isotopic enrichment. For this purpose I applied $^{14}CO_2$ to the illuminated leaf discs in the oxygen electrode and subsequently I fractionated the material and measured sample radioactivity. The experiment revealed no differences between the genotypes in the uptake and distribution of the radiolabel into neutral, cationic and anionic fraction (Figure 15). According to this data the transgenic CS plants are able to assimilate carbon and subsequently synthesize insoluble components, such as cell wall and starch as well as soluble metabolites such as sugars, amino acids and organic acids at comparable rates to the wild type plants.

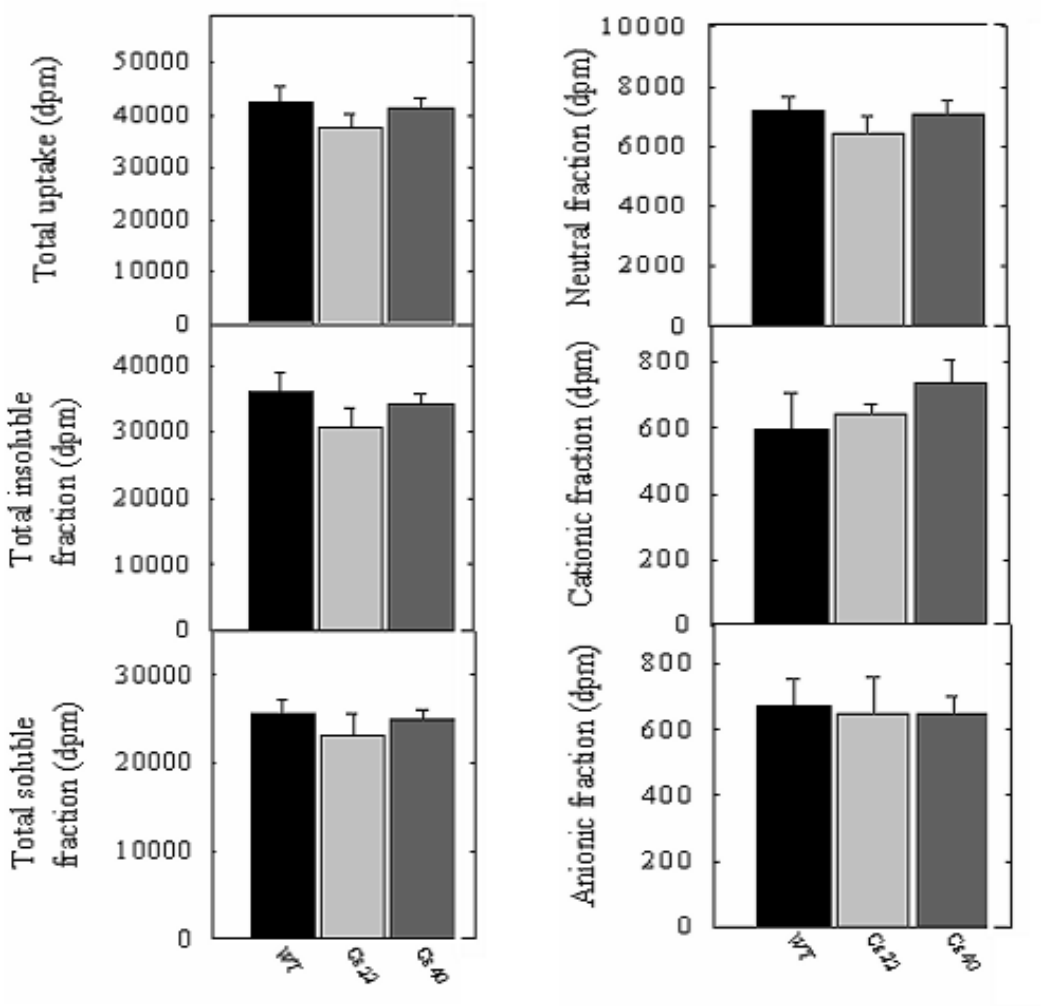


Figure 15: Photosynthetic assimilation and partitioning of carbon in five week old CS transgenic and wild type plants. The mean enrichment ± SE of the radioactive carbon into fractions of leaf discs fed with $^{14}CO_2$ at the onset of illumination in the oxygen electrode was quantified on the basis of six individual plants per line.

The second experiment enabled the direct estimation of the photosynthesis performance in plants *in vivo*. The data revealed complementary results to the previous study, confirming unaltered photosynthetic ability in the analyzed plants. The tomatoes having decreased CS activity displayed a slight tendency toward lower assimilation and transpiration rates in comparison to wild type plants (Figure 16,Figure 17). Nevertheless, the calculated values of chloroplastic electron transport rate obtained by the use of PAM fluorometer were significantly elevated in both tested lines (Figure 18). Similarly, these plants possessed higher respiratory activity in the dark (Figure 19). The differences in the last two parameters were most pronounced in the strongest transgenic line CS40.

To summarize, these data suggest that the down regulation of the mitochondrial citrate synthase had little consequence on the level of photosynthetic performance, with the exception of a large increase in the chloroplastic electron transfer rate and in dark respiration.

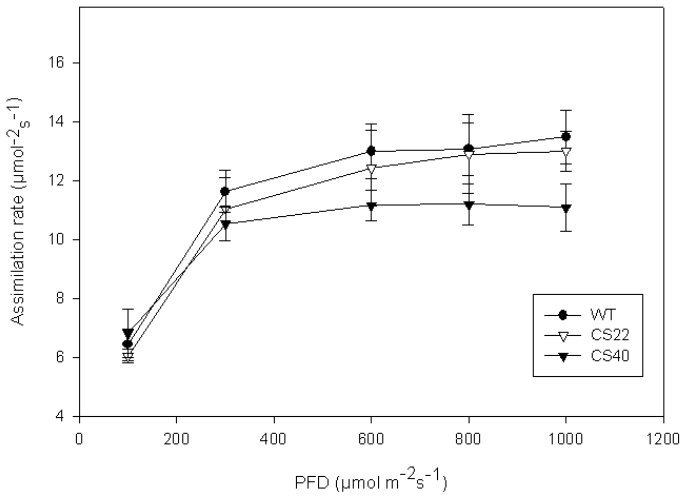


Figure 16: Assimilation rate of illuminated leaves of six week old citrate synthase antisense lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line.

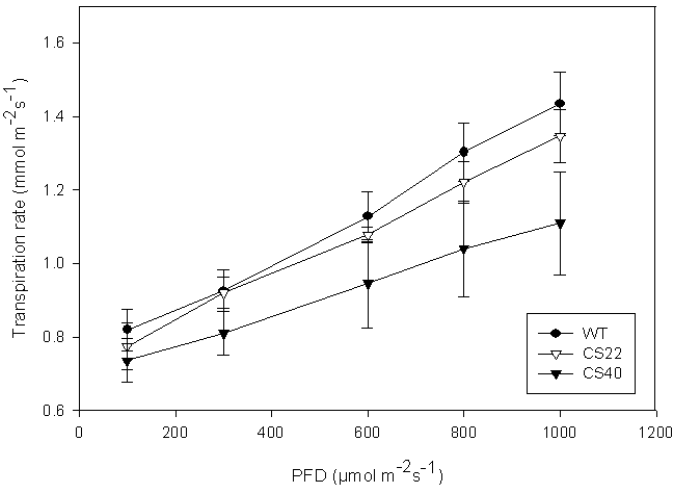


Figure 17: Transpiration rate of illuminated leaves of six week old citrate synthase antisense lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line.

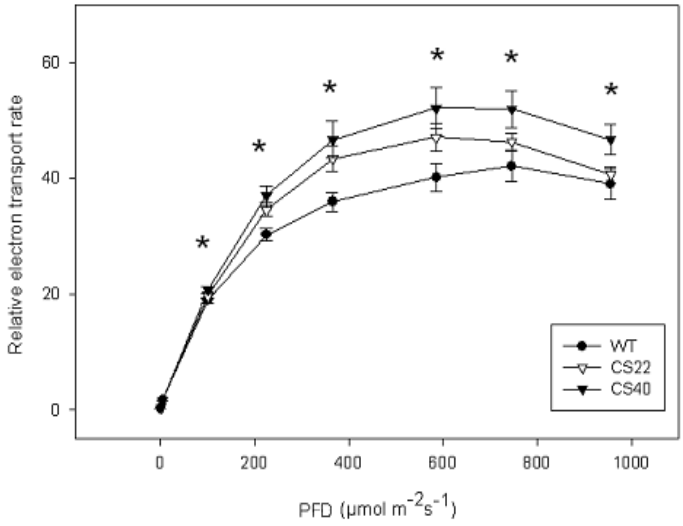


Figure 18: Electron transport rate (ETR) of leaves of six week old citrate synthase antisense lines and wild type plants. In vivo fluorescence emission was measured by the use of a PAM fluorometer at different light intensities. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the Student's t test.

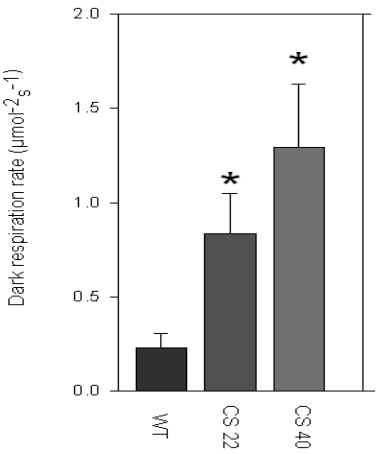


Figure 19: Leaf respiratory activity of six week old citrate synthase antisense lines and wild type plants in dark. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the Student's t test.

I was also interested whether the deficiency in mitochondrial citrate synthase activity would influence TCA cycle activity and respiration. In order to address this question positionally labeled glucoses were fed to the illuminated leaf discs of the transgenic and wild type plants. The released carbon dioxide was captured in KOH trap in hourly intervals and subsequently quantified by liquid scintillation counting. The feeding of glucose specifically labeled at position C3:4 results in the release of CO_2 by the enzymes that are involved solely in mitochondrial respi-

ration (Figure 20), whereas the C1- and C2-labelled glucoses are mainly involved in non-mitochondrial oxidative pathways (Rees and Beevers, 1960).Therefore, the ratio between these values provides valuable information of the relative activity of TCA cycle in comparison to other carbohydrate oxidizing processes within the cell. Interestingly, the remarkably elevated CO_2 absolute values in CS transgenics resulted from C1- and C2- labeled glucose feeding (Figure 21), leading to significant changes in line CS22 (in C1 and C2 position) and line CS40 (in C2 position). When the relative values were analyzed the significantly increased C1/C3:4 and C2/C3:4 ratios for CS22 and CS45 lines led us to the conclusion of impaired mitochondrial respiration ability in the tomato plants exhibiting decreased citrate synthase activity.

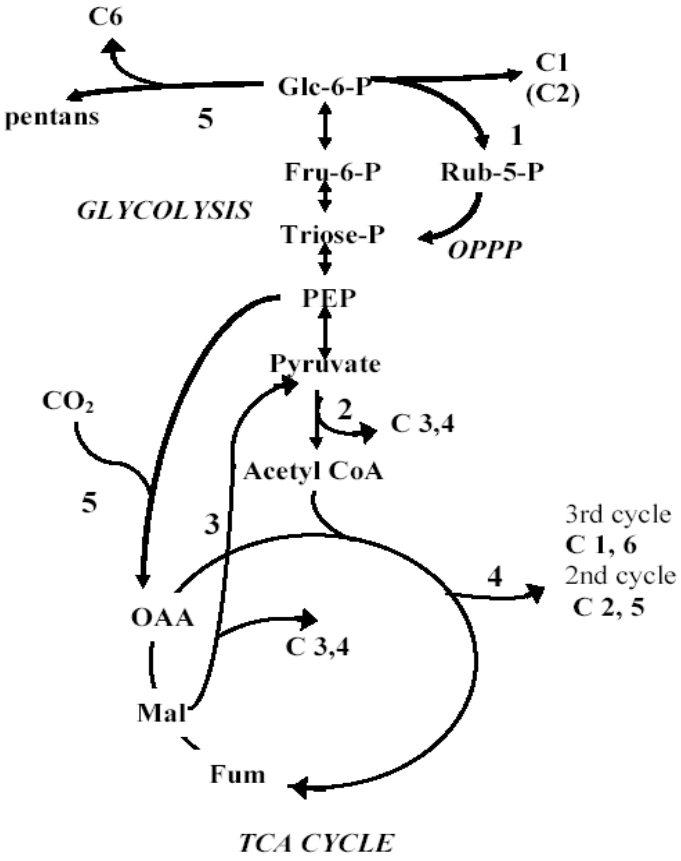


Figure 20: Major pathways of carbohydrate oxidation including specific glucose – derived CO_2 release sites. The decarboxylation reactions are represented by 1-6 and the involved enzymes are: 1: 6-phosphogluconate dehydrogenase; 2: pyruvate dehydrogenase complex; 3: NAD+- malic enzyme; 4: isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase complex; 5: carboxyl lyase.

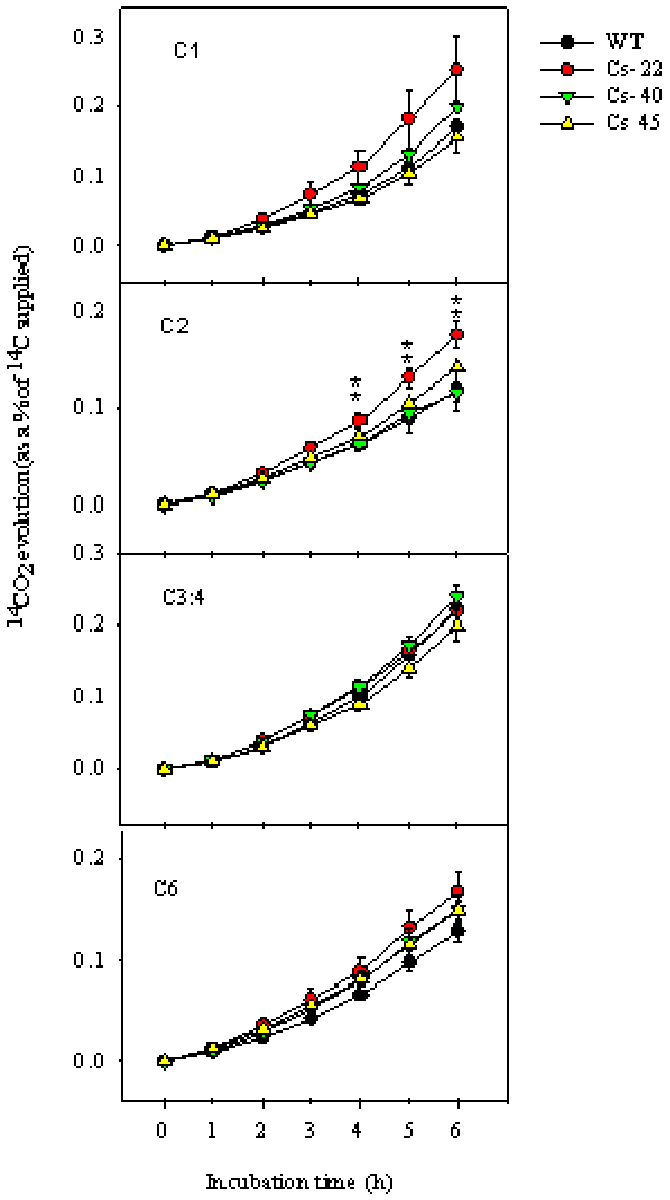
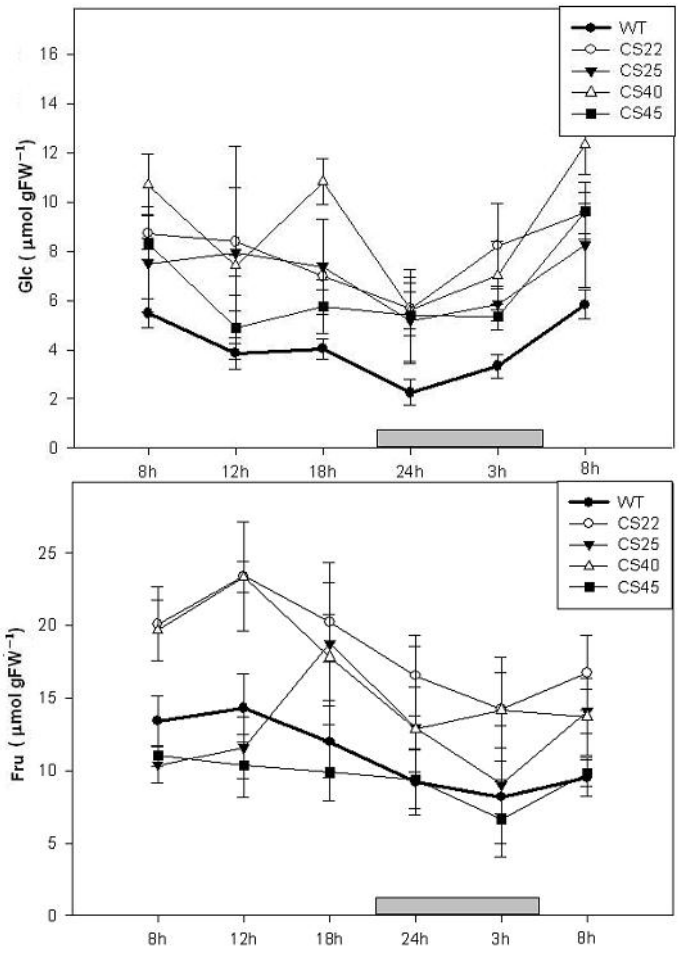


Figure 21: Respiratory performance of five week old CS transgenic and wild type plants presented as $^{14}CO_2$ evolution from the illuminated leaf discs fed with [1-^{14}C]-, [2-^{14}C]-, [3:4-^{14}C]-, or [6-^{14}C]-Glc for six hours. The released radiolabel was captured in hourly intervals in 10% KOH trap and subsequently quantified by liquid scintillation counting. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p< 0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test.

3.3.4 Metabolite profiling of the transgenic CS leaves

3.3.4.1 Leaf carbohydrate content

Having noticed that down-regulation of the mitochondrial citrate synthase activity resulted in only minor changes in plant physiology I next decided to examine the metabolic consequences of the genetic manipulation. Several approaches were applied in order to fulfill this task including the use of highly sensitive GC-MS, HPLC and enzymatic assays techniques. The carbohydrate level, which is known to be highly dependent on C/N interaction was quantified enzymatically at six time points across a day/night period. The diurnal changes of carbohydrate content in leaves revealed a tendency for increased level of soluble sugars with the significantly elevated hexoses in lines CS22 and CS40. Similar, however somewhat less intensive, increasing trend was visible for starch content in the transgenics (Figure 22), probably due to previously reported high variance in the levels of carbohydrates in tomato (Studart-Guimaraes, et al., 2007).



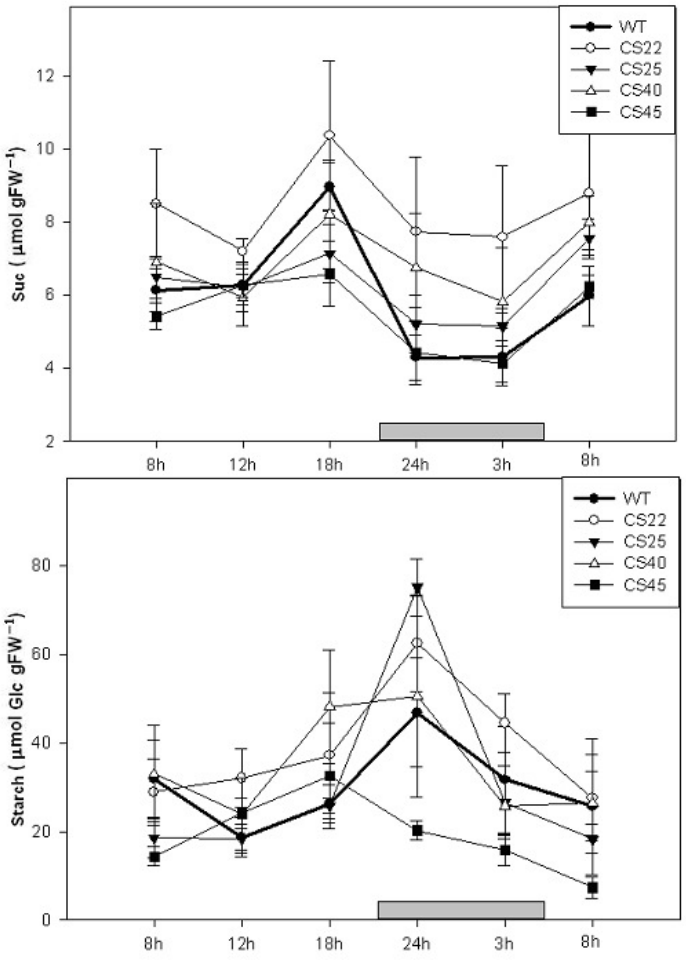


Figure 22: Diurnal changes in carbohydrate content in the source leaves of five week old CS transgenic and control plants. The gray bar indicates the dark period. Values are presented as mean ± SE of determination on six individual plants per line.

3.3.4.2 Leaf nucleotide level

The investigation of sugar nucleotide content by the use of HPLC technique revealed significant changes in both analyzed transgenic lines in comparison to the wild type. The change in ADP-glucose content (which increased) alongside those in the UDP-glucose level (which decreased) were however not accompanied by major changes in other nucleotides (

Table 3). Similarly, the ratio within adenylates and uridinylates remained constant for all genotypes.

Table 3: HPLC - based quantification of nucleotides content in the source leaves of six week old CS transgenic lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. The bold font was used to indicate significantly different ($p<0.05$) values in comparison to wild type as determined by the Student's t test.

Nucleotides level (nmol g FW $^{-1}$)

	WT			CS22			CS40		
ADP-Glc	5.5	±	0.7	**7.3**	**±**	**0.5**	**8.6**	**±**	**1.2**
ATP	30.9	±	8.2	28.8	±	4.6	27.3	±	6.2
ADP	15.8	±	0.6	**18.0**	**±**	**0.8**	14.7	±	0.8
ATP/ADP	2.0	±	0.2	**1.6**	**±**	**0.3**	1.9	±	0.3
∑ adenylates	52.1	±	9.5	54.0	±	5.8	50.6	±	8.2
UDP-Glc	87.7	±	8.3	**63.8**	**±**	**5.7**	**63.7**	**±**	**3.8**
UTP	22.9	±	4.8	21.5	±	1.6	19.1	±	2.4
UDP	4.7	±	0.9	5.1	±	0.8	7.7	±	1.6
UTP/UDP	4.9	±	0.3	4.2	±	0.3	**2.5**	**±**	**0.5**
∑ uridinylates	115.3	±	14.0	90.4	±	8.0	90.5	±	7.9
CTP	5.1	±	0.8	6.1	±	0.9	4.4	±	0.3
CDP	151.3	±	15.7	153.4	±	7.1	151.3	±	12.2
CTP/CDP	0.034	±	0.002	0.04	±	0.002	0.028	±	0.002
GTP	21.6	±	4.0	21.0	±	1.9	21.2	±	1.0
GDP	7.4	±	1.2	6.6	±	1.6	**11.1**	**±**	**1.8**
GTP/GDP	2.9	±	0.3	3.2	±	0.3	1.9	±	0.2

3.3.4.3 GC-MS analysis of primary metabolites in the transgenic CS leaves

I next analyzed the broad metabolic profile of leaf material of the transgenics by utilizing powerful gas chromatography - mass spectrometry (GC-MS) method. Generally, the tomatoes possessing decreased CS activity presented severe alterations on the metabolite level. Surprisingly, there were no dramatic changes in the content of organic acids, although the level of chlorogenate and galacturonate was around 1.5 times increased in the transgenic lines in comparison to wild type (Figure 23). As it would be expected, the citrate level in the citrate synthase – antisensed plants was significantly decreased. Similar transition was observed for citramalate and malate, however the remaining TCA cycle intermediates showed rather opposite tendency. To my surprise the 2-oxoglutarate content was largely elevated in all analyzed transgenic lines, whereas succinate content was significantly increased in CS40. In contrast, the threonate, glycerate, gluconate and glutarate level was slightly decreased. On the basis of GC-MS results I also noticed a slight accumulation of carbohydrates and hexose phosphates in the lines with decreased citrate synthase activity in comparison to wild type plants (Figure 24). Approximately three fold increase in the maltose content was accompanied by significant increase in arabinose, isomaltose, galactose, gentobiose, rhamnose, ribose and xylose. Among the secondary metabolites I noticed around two fold

accumulation of shikimate and dopamine and to a lesser extend tyramine (data not shown). Both, the palmitic and stearic fatty acid content was diminished, significantly for one line CS40 (data not shown).

Interesting changes were observed in the amino acid level in the transgenic lines (Figure 25). Generally, the content of several amino acids involved in nitrogen metabolism such as alanine, beta-alanine, GABA and to a lesser extend glutamate and aspartate was decreased in CS-antisensed lines in comparison to wild type plants. Surprisingly, the major increase within amino acids pool was observed for glutamine and asparagine that were almost six and eight times respectively up regulated in all transgenic lines. Moreover, the CS antisense plants were also characterized by significantly elevated levels of phenylalanine, tryptophan, tyrosine, glycine, and serine, however the branched chain amino acid levels remained unaltered.

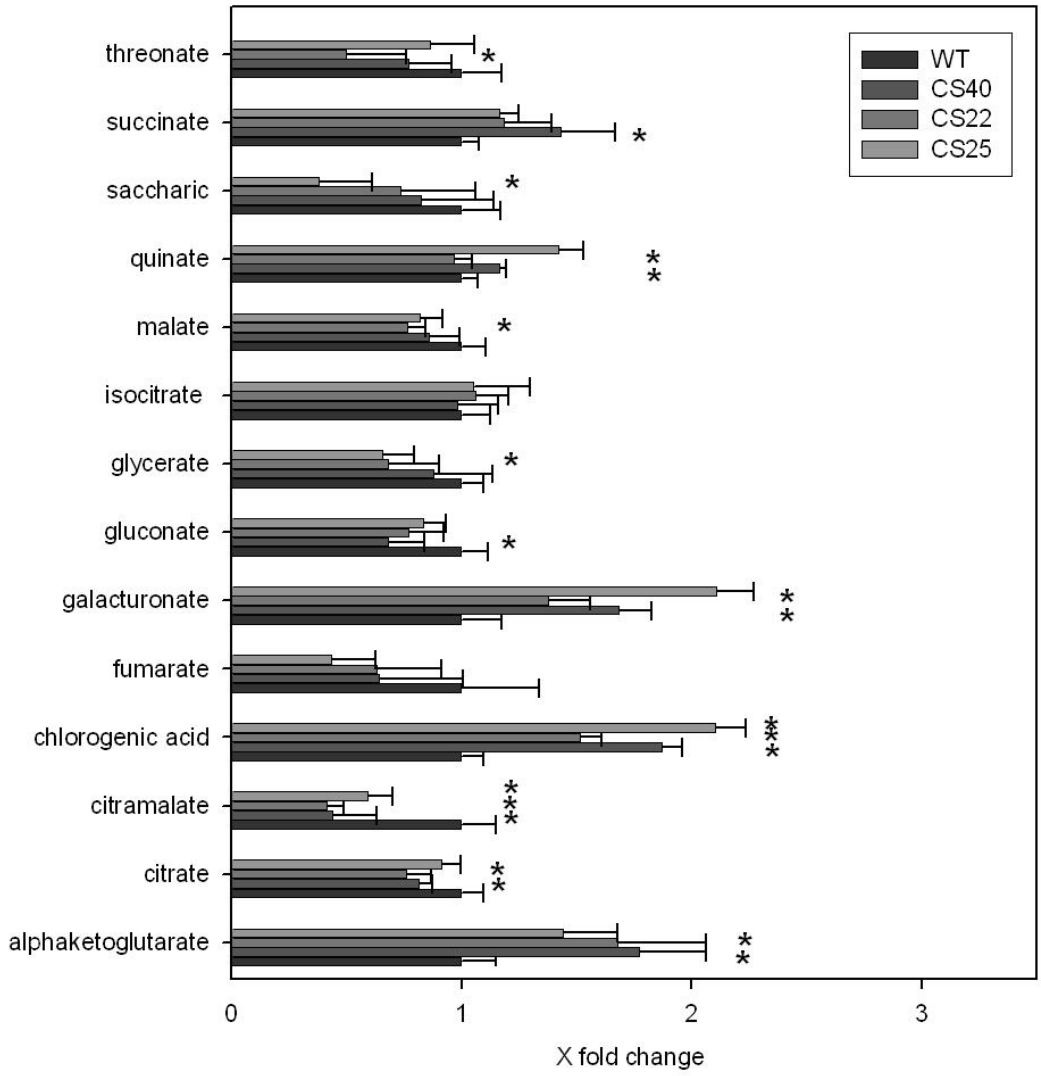


Figure 23: Relative organic acid levels of tomato CS antisense plants obtained by the use of GC-MS technique. Data were normalised to the mean response calculated for wild type control. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test.

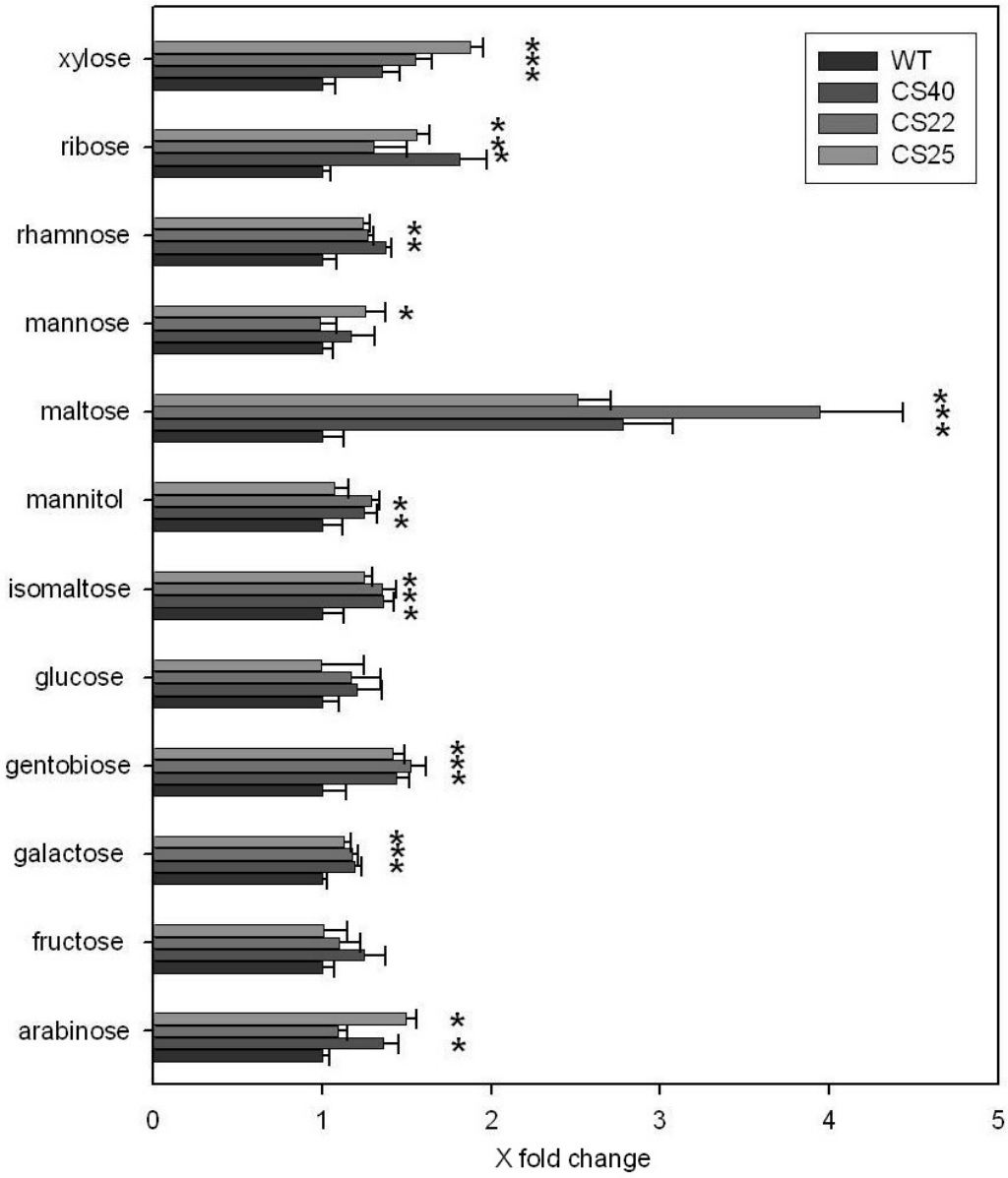


Figure 24: Relative carbohydrate levels of tomato CS antisense plants obtained by the use of GC-MS technique. Data were normalised to the mean response calculated for wild type control. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test.

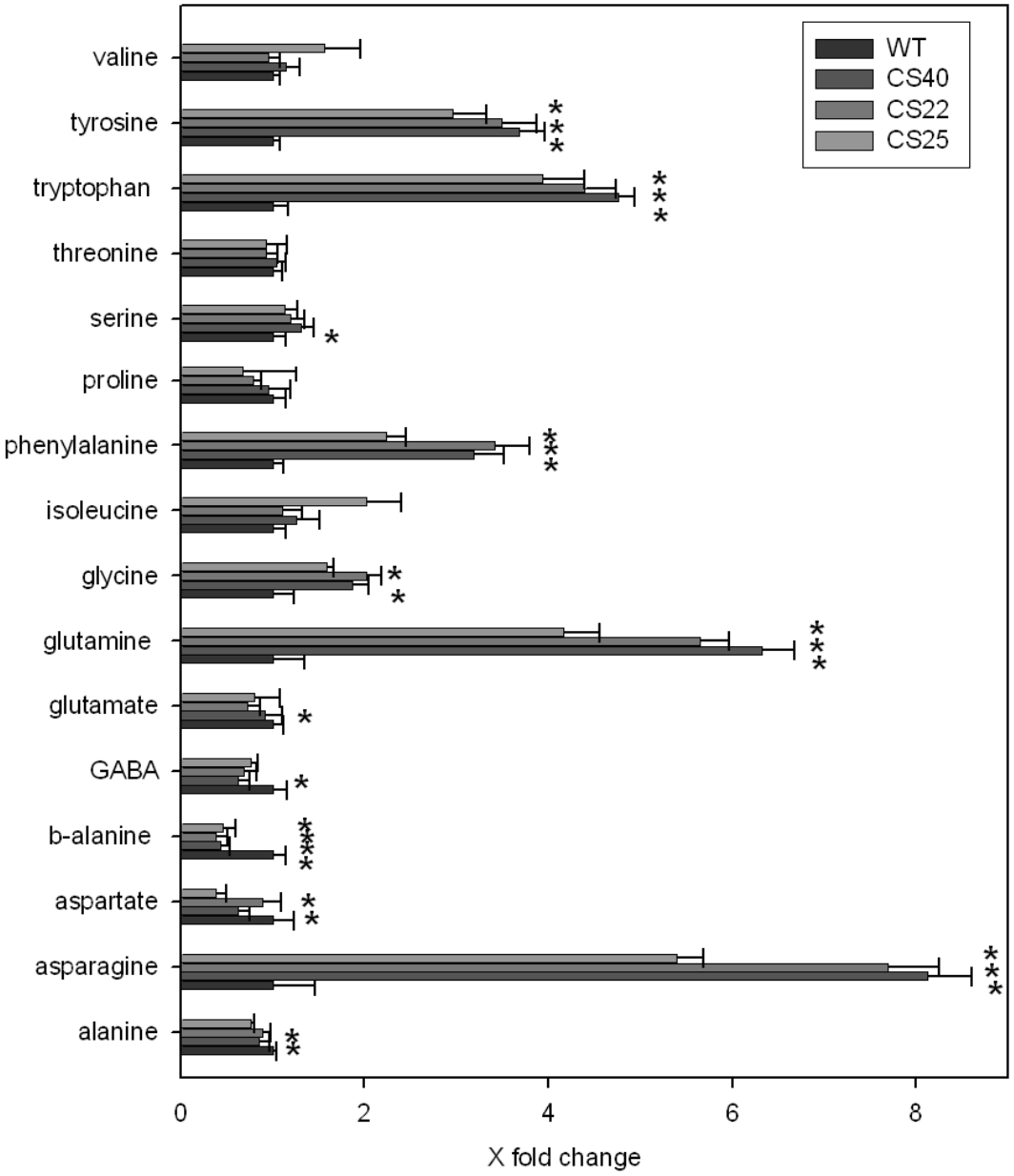


Figure 25: Relative amino acid levels of tomato CS antisense plants obtained by the use of GC-MS technique. Data were normalised to the mean response calculated for wild type control. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test.

3.3.4.4 Diurnal turnover of major amino acids

The metabolic data were subsequently validated by the high performance liquid chromatography (HPLC) – based analysis of diurnal changes in the leaf amino acid content (Figure 26). The samples collected at midday were in agreement with

GC-MS results and confirmed dramatically high concentration of glutamine and asparagines as well as accumulation of other above mentioned amino acids in the transgenic leaves. Interestingly, when the dynamics of these metabolites over time is considered, the differences in amino acid concentrations in the transgenic lines are at their maximum at the end of the day/ beginning of the night after which they return to more or less wild type levels.

To summarize, the decreased level of citrate synthase activity has significantly influenced metabolic profile of the transgenic leaves, altering the concentration of both carbon- and nitrogen- rich intermediates of primary as well as secondary metabolism in tomato.

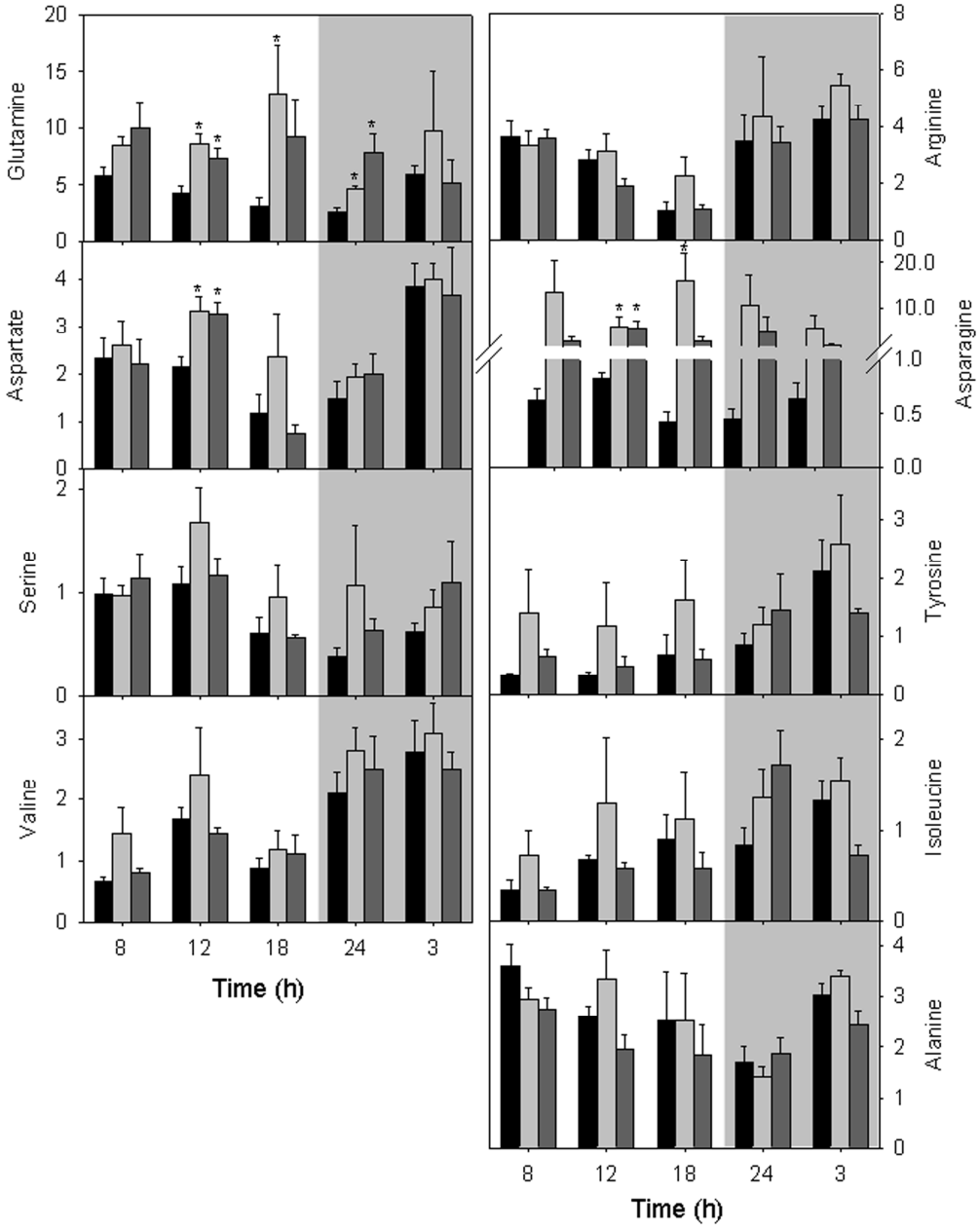


Figure 26: Diurnal changes in leaf amino acids content in leaves of six week old tomato CS anti-sense lines. At each time point, samples were taken from mature source leaves, and the data are presented as µmol gFW^{-1} and represent the mean ± SE of measurements from three plants per genotype. An asterisk indicates significantly different ($p< 0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test. Gray bars, dark period; white bars, light period. The lines used were: WT, black bars; CS22, gray bars; CS 40, dark gray bars.

3.3.4.5 Leaf nitrate level

Since the leaf amino acid content was severely altered in the transgenic plants, I next decided to evaluate nitrogen status in these organs. Although the total protein and leaf nitrogen content in all four transgenic lines resembled wild type level (data not shown), I expected that the down regulation of mitochondrial citrate synthase gene may influence internal nitrate content, which is an important factor of carbon-nitrogen balance within the cells (Foyer and Noctor, 2002). Indeed, spectrophotometrically assayed NO_3 was more than five times elevated in the strongest transgenic lines (CS22 and CS40) and around two times increased in the remaining lines in comparison to wild type plants (Figure 27). The high concentration of nitrate within the leaves resulted presumably from diminished metabolism of nitrate utilizing enzymes which led me towards a theory of lowered nitrogen assimilation performance in the CS transgenics.

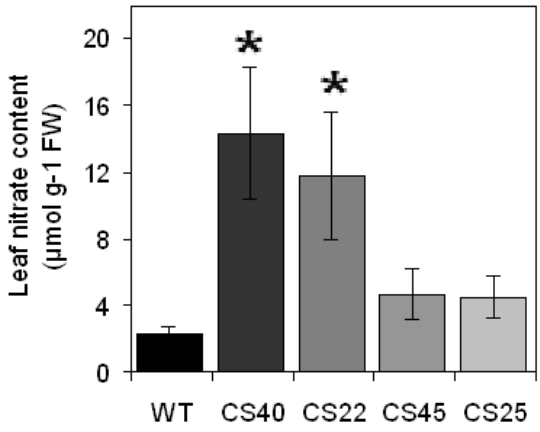


Figure 27: Intracellular nitrate level of source leaves of five week old CS transgenic and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the Student's t test.

3.3.4.6 Photosynthetic pigment content

Finally, I investigated important indicators of nitrogen deficiencies (Gaude, et al., 2007) such as leaf photosynthetic pigments by the use of HPLC technique. Interestingly, the content of leaf pigments was largely decreased in the CS transgenics, supporting the naked eye observation of slightly lighter colour of leaves. The alpha-chlorophyll level was significantly lowered in three transgenic lines in comparison to wild type plants and similar tendency was observed for beta-chlorophyll and beta-carotene content (Figure 28). Among the xanthophylls both neoxanthin and violaxanthin were significantly underrepresented in the leaves of three transgenic lines leading to mild reduction in total xanthophyll cycle intermediates level. Such decrease of light protecting pigments may partially diminish

their function (Bassi, et al., 1997), resulting in higher chloroplastic electron flow under high light stress, however my plants were not subjected to such conditions.

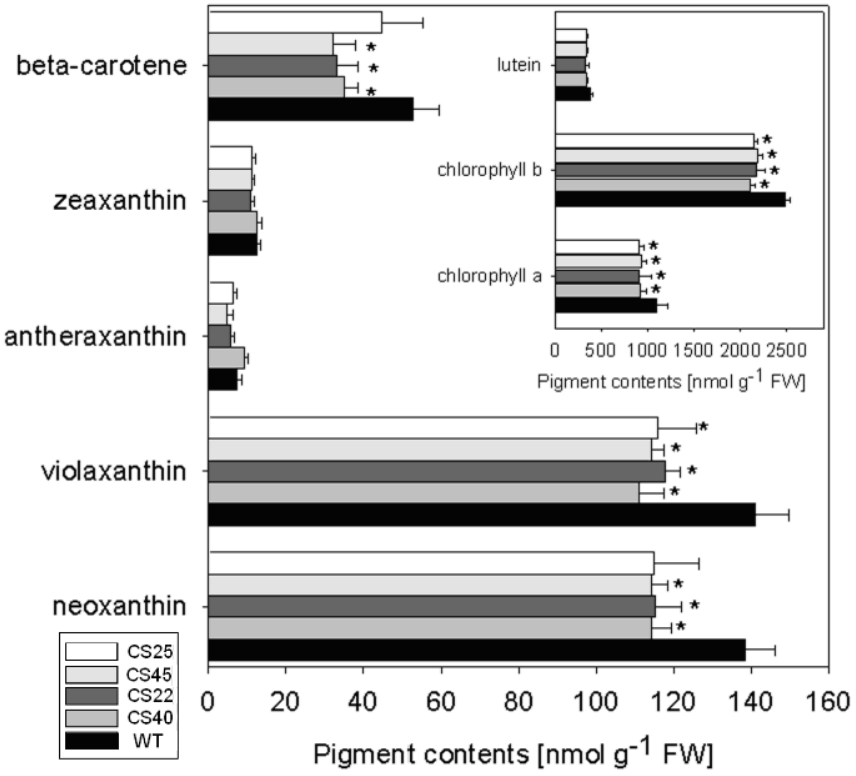


Figure 28: Photosynthetic pigment content of six week old CS transgenic and wild type plants. Samples used were harvested at exactly the same time as those for enzyme determinations presented in Table 4. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p< 0.05$) values in comparison to wild type as determined by the Student's t test.

3.3.5 Measurement of enzyme activities involved in primary metabolism

Having measured citrate synthase activity I next quantified maximal catalytic activities of major enzymes of photosynthetic carbon metabolism in the leaves by the use of a robotized cycling assay (Table 4). The transgenic lines displayed clear decreases in ADP-glucose pyrophosphorylase (in three of the four transgenics), cytosolic fructose-bisphosphatase, phosphoglucomutase, glutamine synthase, the NADP-dependent isoform of isocitrate dehydrogenase and nitrate reductase (in all lines) and NADP-dependent malate dehydrogenase (in two lines). Interestingly, the calculated activation state of the latter enzyme was significantly decreased for CS25 and CS22, hinting towards alterations in the chloroplast redox level (data not shown). In contrast to these findings, the level of acid invertase increased in line CS45, however, changes in these activities were not consistent with the change in citrate synthase activity indicating that they were most likely a pleio-

tropic effect. Activities of fructokinase, hexokinase, glucose 6-phosphate dehydrogenase (G6PDH), NAD-isocitrate dehydrogenase, ATP-dependent phosphofructokinase and succinyl CoA ligase were invariant across the genotypes. Nevertheless, I summarize that the introduced genetic modification has clearly resulted in strong C-N imbalance in tomato leaves leading to pronounced inhibition of several major enzymes involved in both carbon and nitrogen metabolic pathways.

Table 4: Enzyme activities determined in fully expanded source leaves of six week old CS transgenics and wild type plants harvested six hours into the photoperiod. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test.

Enzymatic activities (nmol min^{-1} g^{-1} FW)

	WT	CS22	CS40	CS25	CS45
Acid Invertase	204.45 ± 36.48	203.37 ± 43.08	218.95 ± 35.49	241.51 ± 49.91	410.00 ± 176.12
AGPase	1211.73 ± 22.06	**902.73 ± 78.71**	1217.43 ± 96.19	**890.54 ± 130.04**	**923.49 ± 122.81**
Fructokinase	262.2 ± 25.71	183.56 ± 41.8	237.49 ± 31.66	303.03 ± 22.26	300.2 ± 34.98
Fructose-1,6-bisphosphatase (cytosolic)	140.34 ± 16.73	**89.81 ± 17.35**	**95.05 ± 14.00**	**55.35 ± 4.41**	**51.91 ± 5.28**
Glucokinase	117.81 ± 20.47	92.5 ± 14.66	101.05 ± 19.76	120.3 ± 17.23	114.8 ± 10.56
G6PDH	725.51 ± 168.16	799.79 ± 219.85	618.89 ± 129.08	905.49 ± 230.76	702.04 ± 91.23
NAD-Glutamate dehydrogenase	519.16 ± 23.84	**362.56 ± 33.32**	470.54 ± 42.02	570.36 ± 39.47	555.04 ± 23.26
Fd-GOGAT	2089.89 ± 232.76	**1270.01 ± 233.50**	1351.88 ± 730.00	2048.4 ± 919.99	1572.30 ± 840.00
Glutamine synthetase	1868.23 ± 312.56	**611.12 ± 227.54**	**967.12 ± 238.28**	**356.57 ± 129.11**	**257.81 ± 61.84**
NAD-Isocitrate dehydrogenase	60.44 ± 7.50	62.04 ± 6.33	61.47 ± 11.49	43.42 ± 7.76	42.25 ± 9.45
NADP-Isocitrate dehydrogenase	1032.39 ± 110.07	**600.4 ± 99.24**	**674.01 ± 45.48**	**690.08 ± 99.32**	**711.17 ± 86.66**
NADP-Malate dehydrogenase (initial)	51.31 ± 3.23	**33.31 ± 5.40**	40.64 ± 3.59	**47.6 ± 7.26**	54.03 ± 3.56
NADP-Malate dehydrogenase (total)	110.28 ± 8.79	**78.23 ± 5.58**	89.08 ± 5.74	**113.83 ± 5.89**	113.05 ± 7.48
Nitrate reductase	389.48 ± 33.13	**237.57 ± 47.01**	**206.07 ± 26.42**	**50.17 ± 35.25**	**155.27 ± 49.55**
PEP Carboxylase	745.56 ± 38.31	**528.13 ± 30.99**	**618.41 ± 31.81**	**620.49 ± 20.32**	**621.88 ± 28.12**
Phosphofructokinase (ATP)	379.98 ± 66.26	356.94 ± 84.62	389.05 ± 65.61	284.01 ± 76.69	176.78 ± 38.77
Phosphofructokinase (PPi)	198.47 ± 12.96	**147.47 ± 13.46**	182.95 ± 20.83	**74.27 ± 22.98**	**60.81 ± 13.5**
Phosphoglucose isomerase (cytosolic)	3513.14 ± 167.74	**2652.01 ± 215.8**	**2782.69 ± 179.44**	3695.11 ± 153.25	3472.55 ± 172.16
Phosphoglucose isomerase (total)	5338.27 ± 264.43	**3829 ± 323.21**	**4286.82 ± 218.92**	5297.11 ± 212.37	5175.04 ± 366.01
Phosphoglucomutase	7208.23 ± 314.61	**4570.91 ± 321.14**	**4990.02 ± 267.94**	**6375.39 ± 342.31**	**6082.76 ± 399.16**
Shikimate dehydrogenase	582.86 ± 49.34	**403.21 ± 32.41**	**471.48 ± 44.28**	**415.18 ± 35.33**	**426.8 ± 27.17**
Succinyl-CoA ligase	14.99 ± 2.21	14.29 ± 4.14	13.59 ± 3.17	12.03 ± 2	14.71 ± 3.02
Sucrose Phosphate Synthase	2225.51 ± 168.24	**1584.05 ± 238.06**	**1655.94 ± 172.24**	2036.54 ± 189.08	1953.84 ± 248.9

3.3.6 Transcript profiling of the transgenic CS leaves

The relative transcript level was measured in the tomato leaves by utilizing a real time RT-PCR technique (Czechowski, et al., 2004). The analysis of the expression of all citrate synthase isoforms by the use of specific primers revealed that the transcript level of mitochondrial isoform was decreased in all transgenic lines to a comparable level (Figure 29). Interestingly, one of the peroxisomal citrate synthase mRNA was significantly up regulated in the transgenic tomatoes, probably in order to compensate the low mitochondrial CS activity. However, the transcript content of the other peroxisomal tomato gene was generally unaltered in the transgenic plants.

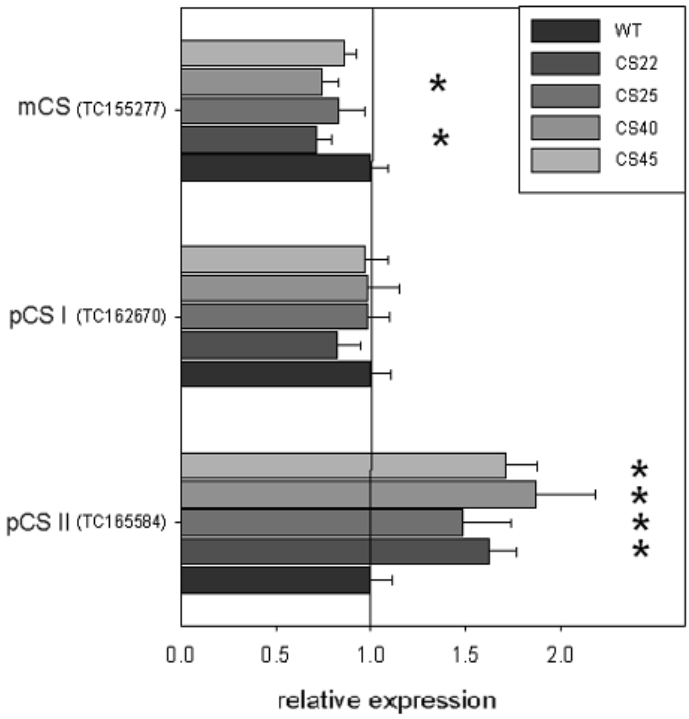


Figure 29: The transcript level of all tomato citrate synthase isoforms determined by RT-RT-PCR technique in source leaves of five week old CS transgenics and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p<0.05$) values obtained for each line in comparison to wild type as determined by the Student's t test. Abbreviations: mCS-mitochondrial CS isoform, pCS – peroxisomal CS isoforms.

The comparison between the differentially expressed genes in the transgenic line CS22 with significantly reduced citrate synthase activity and wild type plants was expanded by performing DNA microarrays. Labeled cDNAs were co-hybridized with the commercially available tomato TOM1 chips. The data normalization was done in R programme (http://www.r-project.org/) and the statistical analysis and visualization was performed in MapMan software (Thimm, et al., 2004; Usadel, et

al., 2005) which allows to conveniently organize huge amount of expression data into functional biochemical pathways presented as BINs. On the BIN level, the Wilcoxon Rank Sum test corrected by Benjamini Hochberg method revealed massive changes in the transcript level of genes involved in both light and dark reactions of photosynthesis, starch and sucrose metabolism, photorespiration, mitochondrial electron transport, aspartate-derived amino acids metabolism and metal handling (Figure 30). The majority of the genes engaged in the above mentioned processes showed increased expression in line CS22 in comparison to wild type level. This result was subsequently confirmed by another statistical tool, the Fischer's test, available in PageMan software (Usadel, et al., 2006) (data not shown). This additionally pointed towards an elevated level of several genes involved in redox regulation, such as glutaredoxins, peroxiredoxins and catalases as well as increased flavonoid synthesis. Both tests also revealed a trend for increased transcription of the genes responsible for protein synthesis and degradation. Surprisingly, a decreased transcript level in line CS22 was observed for the genes composing only three BINS those containing genes encoding carbonic anhydrases, cell wall degradation enzymes and tetrapyrrole synthesis enzymes. The latest one includes also chlorophyll synthatase gene which may provide an explanation for my previous observation of decreased chlorophyll content in the CS-transgenic plants.

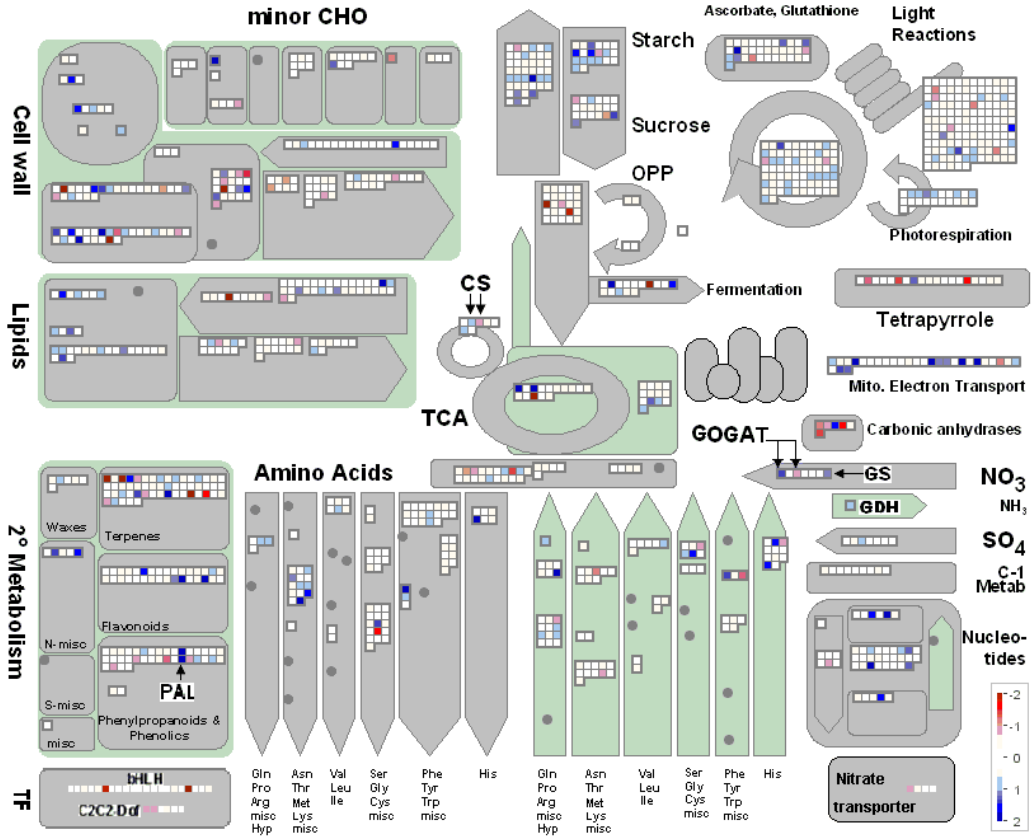


Figure 30: Transcript profiling in leaves. The figure represents x fold increase (in blue) and decrease (in red) in the transcript levels of citrate synthase – antisense line CS22 in comparison to wild type control for genes associated with metabolism and selected transcription factors (TF). The applied colour scale is reproduced in the figure. The data represent mean value of four individual plants for each genotype.

On the single gene level, interesting changes were observed for the genes influencing nitrogen metabolism (Figure 30). Surprisingly, the transcript level of glutamine synthetase, ferredoxin – dependent glutamate synthase and NADH-dependent glutamate dehydrogenase and was more than two fold increased. Oppositely, the NADH-GOGAT isoform and high affinity nitrate transporter were around two fold less expressed in CS22 than in wild type plants. The unexpected inconsistencies between transcript level and enzymatic activities can be partially explained by the absence of several genes on TOM1 chip that offers far less than full genome coverage. Moreover, recent studies indicated the lack of a linear relationship between transcript response and cellular protein levels (Gibon, et al., 2004b).

Among secondary metabolism associated genes a massive elevation of transcript level of phenylalanine - ammonia lyase, a key enzyme in phenylpropanoid biosynthesis pathway that was previously noticed to be induced in nitrogen deficient

tobacco wild type plants (Fritz, et al., 2006b). Interestingly, the level of several transcription factors (TF) that are known to be engaged in the nitrogen metabolism was also altered in CS transgenic plants, for example sixteen fold decrease in the level of bHLH family member of TF that play an important role in nitrate signaling (Lea, et al., 2007). Furthermore a similar, however less dramatic, trend was observed for Dof zink finger proteins that have previously been suggested to be key regulators of carbon-nitrogen interactions in plants (Yanagisawa, 2004). Changes were also present in the level of transcription of genes associated with carbon metabolism with the decreased transcription of several glycolytic enzymes such as cytosolic phosphoglycerate kinase, pyruvate kinase and enolase being accompanied by an elevated transcript level for photorespiratory enzymes.

3.3.7 Quantification of carbon flux between primary C and N metabolites

Having noticed that the down regulation of citrate synthase gene in tomato leads to an imbalance in C/N metabolism I next investigated possible alterations in an internal carbon flow. For this purpose the detached fully developed leaves of two transgenic lines CS22 and CS40 and the wild type plants were fed via the transpiration stream for three hours with 20mM ^{12}C- and ^{13}C- sodium pyruvate under high light conditions in the phytotron. The subsequent metabolite extraction, separation and quantification on GC-TOF were followed by calculation of carbon fractional enrichment factor (CFE) for each metabolite of interest. The unidirectional carbon flux between two metabolites was estimated on the basis of a ratio between the CFE of the product and the precursor, including their concentrations and the time of feeding. The comparison between the carbon flux values obtained for transgenic and wild type plants revealed interesting changes in flux between TCA cycle intermediates (Table 5). As it would be expected, there was significantly lowered carbon flow from citrate to 2-oxoglutarate in both analyzed lines of citrate synthase down regulated plants. Surprisingly, the flax from citrate to succinate was markedly increased in CS22 and CS40 lines in comparison to wild type, suggesting up regulation in the transgenics of another pathway leading the carbon flow directly from citrate to succinate such as glyoxylate pathway. A similar tendency was observed during the analysis of flux from isocitrate to 2-oxoglutarate and succinate. However, the flux between the metabolites acting in the following steps of the TCA cycle (succinate to malate, malate to citrate) was generally at the comparable level for all genotypes of tomatoes. Interestingly, more than five and seven fold increase in the carbon flow from glycine to serine in CS22 and CS40 line respectively was another argument for an elevated activity of photorespiration. Similar, quite dramatic decrease in carbon flow was also observed from malate to aspartate and from 2-oxoglutarate to glutamate in both transgenic lines. Therefore, I conclude that the citrate synthase down regulated plants exhibit an altered dynamics of metabolic processes than control plants. The modification of citrate synthase activity in tomato leaves resulted in decreased performance of early steps of TCA cycle causing restriction in pyruvate derived fluxes through citrate synthase and diminishing nitrate assimilation process. The involvement of

alternative pathways sustained or even enhanced succinate supply to maintain mitochondrial electron transport chain.

Table 5: Estimated unidirectional carbon exchange rates in CS transgenics and wild type plants. The whole, detached source leaves of greenhouse grown plants were transferred to high light conditions and fed with [U-^{13}C] pyruvate via the petiole. The synthetic flux represents a ratio between the carbon fractional enrichment factors (CFE) of precursor and product.

Synthetic flux (precursor) (µmol g FW^{-1} h^{-1})

	WT			CS22			CS40		
2-OG (citrate)	0.249	±	0.031	**0.131**	±	**0.001**	**0.117**	±	**0.017**
succinate (2-OG)	0.079	±	0.0026	**0.101**	±	**0.0065**	0.091	±	0.0112
2-OG (isocitrate)	0.772	±	0.084	**0.424**	±	**0.002**	**0.250**	±	**0.010**
succinate (isocitrate)	0.181	±	0.015	**0.363**	±	**0.015**	0.286	±	0.164
succinate (citrate)	0.061	±	0.009	**0.105**	±	**0.006**	**0.129**	±	**0.008**
malate (succinate)	1.175	±	0.104	**0.757**	±	**0.093**	0.953	±	0.142
citrate (malate)	0.115	±	0.016	0.094	±	0.007	0.081	±	0.016
Serine (glycine)	0.0004	±	0.00015	**0.0022**	±	**0.00040**	**0.0029**	±	**0.00033**
2-OG (glutamate)	0.150	±	0.012	**0.050**	±	**0.0022**	**0.032**	±	**0.0002**
glutamate (2-OG)	0.282	±	0.0244	**0.169**	±	**0.0076**	**0.224**	±	**0.0017**
aspartate (malate)	0.085	±	0.0049	**0.024**	±	**0.0008**	**0.020**	±	**0.0011**

3.4 Discussion and conclusion

The investigation of the effect of decreased activity of citrate synthase on physiology and metabolism of tomato plants was performed on stable transformants carrying mitochondrial isoform of endogenous CS gene in the antisense orientation. The effective and constant transcription of insert controlled by 35S promoter resulted in diminished CS performance on both enzyme and mRNA level throughout all plant organs (data not shown). Nevertheless, the genetic modification had little effect on plant phenotype and photosynthetic performance. This is surprising when keeping in mind that correct functioning of respiratory pathway is required to sustain optimal rates of photosynthesis (Igamberdiev, et al., 2001a; Igamberdiev, et al., 2001b; Raghavendra and Padmasree, 2003; Sweetlove, et al., 2006; Nunes-Nesi, et al., 2007b). Nevertheless, the inhibition of the CS enzymatic activity in the following eukaryotes *Saccharomyces cerevisiae* (Jia, et al., 1997), *Solanum tuberosum* (Landschutze, et al., 1995b), *Podospora anserina* (Ruprich-Robert, et al., 2002) resulted in lack of significant respiratory defects and no vegetative growth phenotype. Moreover, the transgenic CS potato plants presented unaltered photosynthetic performance. All these features were observed in CS antisense tomatoes described here. Additionally, the above mentioned mutants, except for yeasts, suffered from fertility deterioration that did not appear to a great extent in tomato plants. It is however perfectly understandable when bearing in mind that the inhibitory effect on flowering of Landschuetze's plants was strictly dependent on the level of residual enzymatic activity, resulting in disintegration of

ovaries emerging only in few severely inhibited potato lines! This phenomenon is also exemplified in my plants by the lowest flower production in the strongest transgenic line CS22. In comparison to the transgenic potato with a mere 6 % rest activity produced by Landschuetze and co-workers (1995b) the selection process recovered only tomato plants with mild reductions in CS activity, which is astonishing. It may be explained by either existence of a second, unidentified mitochondrial isoform of CS in tomato, or by peroxisomal isoform providing a higher contribution to the total activity in tomato than potato, however the extreme similarity between genome and physiology of the two *Solanaceae* species does not support these hypotheses. Interestingly, as revealed by the RT-PCR data presented here, the upregulation of the transcript level of peroxisomal citrate synthase arose in the transgenic tomato leaves. The higher activity of this isoform may at least partially compensated the metabolic and physiological changes caused by the down-regulation of the mitochondrial CS. It could also mask some of effects of the genetic manipulation, including overstating the total citrate synthase activity, on which basis the plants were screened. In my hypothesis (Figure 31), the over-activated peroxisomal CS isoform produces citrate, perhaps during operation of the glyoxylate cycle. Subsequently, citrate is either further metabolised within the peroxisome or exported and taken up into the mitochondria, either in the form of citrate or isocitrate, in counter exchange for oxaloacetate via the operation of a previously characterized carboxylic acid transporter (Picault, et al., 2002). It is possible that the oxaloacetate exported from the mitochondria could subsequently be taken up by the peroxisome to maintain the activity of the peroxisomal citrate synthase. However, whether this occurs *in vivo*, or if it is rather first converted to aspartate before import, remains contentious (Mettler and Beevers, 1980; Pracharoenwattana, et al., 2005). Nevertheless, there is an increased and presumably varied from the one occurring in wild type plants flow of metabolites between organelles, as confirmed by an elevated transcript level of both mitochondrial and peroxisomal carrier genes in CS transgenic plants.

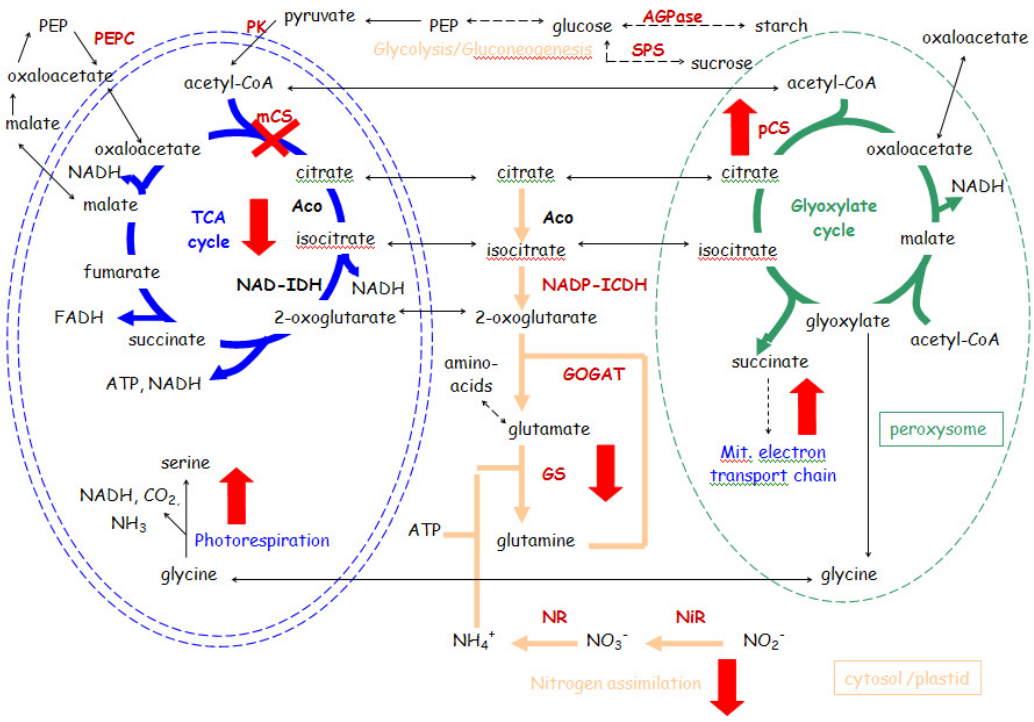


Figure 31: Hypothetic alterations of the metabolic and transcript level revealed in CS antisense tomato plants as the compensatory mechanism. The transgenic leaves posses decreased mitochondrial citrate synthase activity that results in diminished flux through the TCA cycle and changes in reductant content within this organelle. The compensatory up regulation of peroxisomal CS isoform produces citrate for supplying nitrogen assimilation pathway with carbon skeletons in the form of 2-oxoglutarate, generated either by concerted action of cytosolic and/or mitochondrial Aco and IDH/ICDH enzymes. The decreased flux through this pathway combined with shortages in citrate production results in diminished N assimilation ability which is manifested by limited activity of responsible enzymes and accumulation of 2-OG. (see full description in text). Red arrows pointed down represent decreased flux through TCA cycle and nitrogen/ammonium assimilation pathways performance. Red arrows pointed up stand for upregulation of peroxisomal CS, increased photorespiration and mitochondrial electron transport chain. Enzymes marked in dark-red colour, that is CS, NADP-ICDH, PK, PEPC, NR, NiR, GS, GOGAT, AGPase, SPS are known to respond to alterations in nitrogen supply (Scheible, et al., 1997a; Stitt and Scheible, 1999) and their activity was significantly decreased in CS transgenic plants.

The existence of an bypass mechanism that efficiently compensated for genetically imposed system instability may be responsible for considerable shifts in metabolism of CS antisense plants. Most dramatic amongst these was the concerted down regulation of enzymes of nitrate assimilation. The level of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydroganse (GDH), as well as phosphoenolopyruvate carboxylase (PEPC), NADP-dependent isocitrate dehydroganse (NADP-ICDH) and ADP- glucose pyrophosphorylase (AGPase) significantly dropped down as a result of lowered mCS activity. The alterations in transcript level and activity of primary and secondary nitrogen assimilatory pathway enzymes together with carbon metabolizing enzymes

was frequently observed in response to limited nitrogen supply, sugar starvation or changed light-dark regimes in multiple experiments (see for example Stitt, 1999; Coruzzi, 2003: for a review on Arabidopsis mutants). According to Scheible, Krapp and Stitt (2000) the performance of NR, PEPC, CS and NADP-ICDH in tobacco leaves were reciprocally dependent and co-regulated in order to tune organic acid derivation for nitrogen assimilation requirement. The results obtained for tomato plants with limited mitochondrial citrate synthase activity and lowered flux through TCA cycle, presenting massive down regulation of nitrate assimilation enzymes activity in spite of growing in nitrogen sufficient conditions, support this theory. Analysis of mRNA levels by microarray in CS transgenics revealed that several transcripts involved in nitrogen metabolism were repressed, however this trend was not universal suggesting that the reduction in nitrate assimilation was, at least in part, mediated at the post-translational level. Apart from the decrease in genes coding N associated enzymes and transcription factors, the transcript level of high affinity nitrate transporter was decreased, probably due to accumulation of nitrate in vacuole and its lowered metabolic use and motility. Among secondary metabolism associated genes there was a massive elevation of transcript level of phenylalanine - ammonia lyase, a key enzyme of phenylpropanoid biosynthesis pathway that was previously shown to be induced in nitrogen deficient tobacco wild type plants (Fritz, et al., 2006b).

In CS antisense plants, the evaluation of steady-state metabolite levels was in keeping with a restriction in nitrate assimilation. Firstly, the level of nitrate was elevated in the transgenic lines, although it should be noted that the total leaf nitrogen content was unaltered in the transgenics. Secondly, the levels of several amino acids were reduced in the transformants. Thirdly, the increased levels of starch and soluble carbohydrates and the decreased levels of photosynthetic pigments observed in the transgenic plants are typical for reduced rate of nitrate assimilation (Fritz, et al., 2006a; Gaude, et al., 2007). Despite the clear restriction in nitrate assimilation, the levels of both asparagine (Asn) and glutamine (Gln) were significantly increased in the CS transformants. The accumulation of these metabolites under nitrate limitation is not without precedence since Orsel and coworkers (2006) reported a similar observation in *Arabidopsis thaliana*. Furthermore, an increase in Asn and Gln was observed in plants grown during extended night conditions and dark-induced senescence (Ishizaki, et al., 2005; Gibon, et al., 2006; Fahnenstich, et al., 2007). Analysis of changes in the diurnal levels of asparagine, aspartate and glutamine revealed that they were less different at the end of the dark period - a fact that supports my hypothesis that these differences are related to the changes in nitrate assimilation seen in these lines. Furthermore the data would seem to suggest that this increase may be due, at least in part, to an increased protein degradation and differential amino acid mobilization within the transformants. Indeed, there is a clear upregulation of genes associated with protein degradation in the transgenic lines and in the rate of metabolism of the high-N amino acids, suggesting altered rates of protein turnover in CS transgenic plants.

The recorded reduction in flux through mitochondrial steps of the TCA cycle could lead to a reduced mitochondrial ATP and NAD(P)H production. It would explain the severe apparent decrease in the *in vivo* activity of nitrate reductase since both ADP and NAD have been reported to inhibit the activity if this enzyme *in vitro* (Campbell and Smarrelli, 1978; Maldonado, et al., 1978). Moreover, higher availability of reductans that were utilised to the lower extend in CS antisense plants suffering from limited nitrogen assimilation process could instead supply strongly up regulated mitochondrial electron transport chain, since the two pathways were proposed to compete for cellular NADH pool (Dutilleul, et al., 2005). The internal carbon flow measurement has shown that, regardless of limited flux through the 'initial' steps of TCA cycle, a sufficient substrate supply to the electron transport chain is sustained or even increased in the CS antisense plants. Additionally, recent studies on mitochondrial respiratory mutants (Sweetlove, et al., 2006) pointed out that higher flux of electrons into the mitochondrial respiratory chain may originate from leaf photorespiration. That finding stays in great agreement with the increased rate of the latter process in CS antisense tomatoes, as deducted on the basis of up regulation of majority of the pathway genes, followed by an elevated content of steady state metabolites and increased carbon flux between glycine and serine. Photorespiration, traditionally thought as a wasteful process shared between multiple organelles, was proposed to serve for a production of amino acids for different metabolic pathways (Rachmilevitch, et al., 2004), and therefore influence content of glycine, serine, glutamine and asparagine in plant leaves (Noctor, et al., 1999). Indeed, the level of all these amino acids was significantly increased in CS antisense plants, however it may result from multiple physiological processes, since these metabolites were proposed to be markers of both photorespiration (Gly, Ser) and nitrogen assimilation (Gln, Asn) (Foyer, et al., 2003). It is therefore more believable that the increased photorespiration was rather playing a protectory role from photoinhibition (Kozaki and Takeba, 1996), especially in leaves of CS antisense plants that possess lowered content of light protecting xanthophyll pigments. The decreased absorption of light due to limited chlorophyll level and increased thermal dissipation due to alterations in pigment content, both similar to the changes observed in my CS transgenics were found in spinach leaves as a result of nitrogen deficiency (Verhoeven, et al., 1997). The malfunction of insufficiently abundant light protecting pigments in CS antisense plants was presumably responsible for significantly elevated chloroplastic electron transport flow rate in leaves.

The data I present here are consistent with other studies in demonstrating a clear role for the peroxisomal production of citrate, in conjuncture with the operation of the mitochondrial TCA cycle, in respiration of acetyl units from peroxisomal beta-oxidation of fatty acids (Hooks, 2002; Pracharoenwattana, et al., 2005). They confirm previous observations that peroxisomal citrate synthase activity is detected in a wide range of tissues and not only those in which the glyoxylate cycle is active (Cornah and Smith, 2002; Pracharoenwattana, et al., 2005) . Additionally, my results revealing up regulation of pCS combined with limited flux through TCA

cycle and lack of major phenotypic deformations in tomatoes stay in agreement with the data obtained for yeast mCS mutants, in which gCS activity failed to fully substitute mitochondrially localised isoform for TCA cycle maintenance, however was sufficient to prevent cells from death and glutamate auxothrophy (Lee, et al., 2006). It can not also be excluded that the increased formation of reactive oxygen species (ROS) that appeared in the *S. cerevisiae* mutants may occur in CS transgenic leaves, as deduced from significant accumulation of antioxidants, such as alpha and gamma tocopherols in transgenic tomatoes (data not shown) and decrease in chlorophyll content. It has been recently demonstrated by Doermann (2007) that free phytol coming from chlorophyll breakdown might directly be salvaged for tocopherol biosynthesis, which is required for antioxidant protection in oxidative stress. The lipid peroxidation, membrane damage and pigment loss that emerge under these conditions can be prevented by accumulation of gamma-tocopherol, providing increased desiccation tolerance in tobacco plants (Abbasi, et al., 2007). I can therefore hypothesize that upregulated pCS and glyoxylate cycle activity predicted in CS transgenics led to increased lipid oxidation and decrease in fatty acids that was partially prevented by elevated tocopherol biosynthesis. Nevertheless, my data confirm presumable protective role of this enzyme that emerges in variety of stress conditions, as proposed by (Lee, et al., 2007).

The typical response of plants submitted to N starvation includes accumulation of sugars, likely due to limited use of sucrose for amino acid synthesis (Stitt and Krapp, 1999). Several studies have proven sugar and starch accumulation combined with lowered nitrogen assimilation (Scheible, et al., 1997b; Geiger, et al., 1998; Geiger, et al., 1999; Matt, et al., 2001b: and 2001a), because inorganic nitrogen is required to utilize carbohydrates for multiple processes, such as growth, photosynthesis and provision of reducing equivalents, ATP and carbon skeletons for amino acid and nucleotides synthesis. Under N deficient conditions, both Arabidopsis wild type and PII mutant leaves were characterized by higher hexose, sucrose and starch level (Ferrario-Mery, et al., 2005). That observation is in keeping with the increased carbohydrate content in nitrate assimilation impaired CS antisensed tomatoes. The approximately three fold increase in leaf maltose content may imply a higher rate of starch degradation in transgenic lines. This finding is somewhat surprising when considering lowered AGPase activity and high ADP-glucose level together with high starch content, however increased transcript level of genes involved in both starch synthesis and mobilization provides an explanation suggesting altered control of starch metabolism in CS transgenics. Moreover, it should be kept in mind that AGPase activity is redox regulated and responds to sugar level (Kolbe, et al., 2005), whereas CS transgenic leaves are likely to posses modifications in the cellular reduction state. The changes in hexose and starch level found in CS antisense leaves were additionally accompanied by significant increase in arabinose, isomaltose, galactose, gentobiose, rhamnose, ribose and xylose, which stay in agreement with lower levels of UDP-glucose, a principal precursor of the biosynthetic pathway, that these lines display. Furthermore, it is probable that the increased sucrose level in plants characterized by upregulated

peroxisomal CS activity resulted from elevated gluconeogenesis. The net production of sucrose from fatty acids via glyoxylate cycle is consistent with significantly altered steady state level of these metabolites in transgenic lines, although whether such scenario can occur in leaves remains ambiguous.

Changes in nutrient supply, including sugar and nitrogen availability lead to coordinated reprogramming of carbon and nitrogen primary metabolism (Fritz, et al., 2006a; Fritz, et al., 2006b; Osuna, et al., 2007), that subsequently affects content of secondary metabolites that take part in signaling and defense against variety of stress sources. Although the majority of amino acids decreased in my CS transgenics, I quantified significant accumulation of nitrogen containing metabolites, such as Phe, Trp, Tyr and Gly, the well known precursors of alkaloids, phenylpropanoids and other secondary metabolites (Herbert, et al., 1992). The carbon-nutrient balance hypothesis (Coley, et al., 1985; VanDam, et al., 1996) postulates that secondary metabolism is directed towards carbon-rich metabolites in nitrogen-limited plants and nitrogen-rich metabolites in carbon-limited plants, however the accuracy of this hypothesis is not known, since multiple studies have both confirmed and contradicted it (for detailed review see Fritz *et al*., 2006a). Similarly to the results obtained by Fritz and co-workers (2006a; 2006b) on nitrate deficient wild type tobacco plants, my studies performed on CS antisensed tomato leaves suffering from limited nitrogen assimilation process have shown elevated level of carbon-rich phenylpropanoids (chlorogenic acid, caffeic acid) and the precursors of the biosynthetic pathway (Phe, Tyr, shikimate), therefore confirming carbon-nutrient balance hypothesis, at least partially since nitrogen-rich metabolites were not quantified in these plants.

In summary, the tomato plants down regulated in the performance of mitochondrial citrate synthase displayed essentially no visible phenotypic alteration from the wild type and relatively few changes in photosynthetic parameters, however they were characterized by decreased relative flux through the TCA cycle and mildly impaired nitrate assimilation. The alteration in the carbon deriving pathway resulted in decrease of maximal catalytic activities of several enzymes involved in primary metabolism as well as multiple changes in the transcript profile in leaves. As revealed by RT-PCR data, the lack of CS activity was partially compensated by upregulation of peroxisomal CS isoform, leading to major alteration in primary and secondary metabolism. Generally, the reprogramming of C and N metabolism following the genetic modification of transgenic tomatoes that occurred on both metabolite and transcript level stays in agreement with current knowledge of regulation of the complex system response in plants.

4 Analysis of the function of mitochondrial and cytosolic isocitrate dehydrogenases on tomato leaf metabolism.

4.1 Introduction

Isocitrate dehydrogenase catalyses the first oxidative decarboxylation reaction of the TCA cycle, yielding 2-oxoglutarate and CO_2 from isocitrate in a two-step reaction. On the basis of cofactor-dependency (NAD or NADP) two types of enzymes can be distinguished. The NAD^+-requiring enzyme (EC 1.1.1.41) is located exclusively in the mitochondrial matrix, whereas the $NADP^+$- dependent isoforms (EC 1.1.1.42) has been isolated from a range of organelles, including chloroplasts (Galvez, et al., 1994), mitochondria (Galvez, et al., 1998), peroxisomes and the cytosol (Galvez, et al., 1996). The mitochondrial NAD-isocitrate dehydrogenase (IDH) is tightly regulated by substrate availability (isocitrate, NAD^+, Mg^{2+} / Ca^{2+}), product inhibition (by NADH and 2-oxoglutarate) and competitive feedback inhibition (by ATP). It is believed that the NAD-dependent mitochondrial isoform catalyses irreversible reaction, in contrast to NADP-requiring enzymes that are able to operate in both directions (Igamberdiev and Gardestrom, 2003). The cytosolic NADP-dependent isoform was shown to be inhibited *in vitro* by the reaction products as well as citrate, glyoxylate and oxaloacetate (Rasmusson and Moller, 1991; Hodges, et al., 2003; Igamberdiev and Gardestrom, 2003). Given the instability and relatively low abundance of NAD-IDH enzyme in plant tissues, it has to date been purified and characterised only in etiolated pea seedlings (McIntosh, 1997). These studies revealed two biochemically distinct subunits of 47 kDa, whose activity was associated with mitochondrial matrix and membrane fraction, respectively. In contrast to the homodimeric structure of the NADP-requiring isoforms, the heteromeric organization of the mitochondrial IDH is based on the existence of the one catalytic and two regulatory subunits, which seem to be conserved between animals and plants but not yeasts (Lancien, et al., 1998). Further studies revealed that out of two catalytic and three regulatory subunits that exist in *Arabidopsis thaliana*, at least one of each kind is necessary to generate functional enzyme *in planta* (Lemaitre and Hodges, 2006). Similar composition of the mitochondrial isoform was proven for other higher plant species, however the number of IDH encoding genes varies between three in rice (Abiko, et al., 2005b) and tobacco (Lancien, et al., 1998) up to six (five transcribed and one pseudogene) in Arabidopsis. Although four genes of the latter were expressed in all plants organs and only one exhibited pollen-specific expression, the transcription level of IDH isoforms differs between tissues, presumably reflecting the specific function of each isoform.

Among all NADP-requiring isocitrate dehydrogenase isoforms, the cytosolic one has the highest activity and has additionally been purified (see for example Gal-

lardo, et al., 1995; Galvez, et al., 1996; Palomo, et al., 1998), intensively studied and well characterised in plants. It was shown to be responsible for 95% of total NADP-ICDH activity in green tobacco leaf tissue (Galvez, et al., 1996) and proven to be the predominant isoform in leaves of at least fifteen plant species (Chen, 1998). According to recent studies, *Arabidopsis thaliana* contains three genes encoding cytosolically located isoform (Leterrier, et al., 2007). Although the isolated potato NADP-ICDH gene was active in all plants organs, the highest expression of the cytosolic isoform was exhibited in green tissues, flowers and roots (Fieuw, et al., 1995). Given the preferential expression of this isoform in mature leaf veins, the enzyme was believed to be involved in cycling, redistribution and export of amino acids, apart from the primary function in GS/GOGAT – dependent nitrogen assimilation. The latter role was initially proposed by Chen and Gadal (1990) as a part of cytosol localized supporting pathway that derives carbon skeletons for ammonium assimilation in particular circumstances when TCA cycle activity is decreased due to specific light-dark regime. It was therefore surprising to find that both potato (Kruse, et al., 1998) and tobacco (Galvez, et al., 1999) plants maintaining only 6-10% of ICDH activity showed no growth or developmental phenotype. However, limited cytosolic ICDH activity was also assigned to improve phosphorus scavenging and therefore promote faster growth of carrot mutant lines due to higher than in wild type cells excretion of citrate (Kihara, et al., 2003).

The activity of the chloroplastic isoform remains at low level in etiolated tissues but can be induced by the transition of the plants from the darkness to the light conditions. The finding gave rise to the theory that this isoform may together with the cytosolic pentose phosphate pathway play an important role in the provision of the NADPH required for plant growth, (Galvez, et al., 1994). The studies of Popova (2002) provided an interesting supposition that the chloroplastic isoform may also contribute to the coordination of carbon and nitrogen metabolism. It was shown that the *in vitro* enzymatic activity of the chloroplastic enzyme was strongly inhibited by the glutamate, whereas the cytosolic ICDH activity was greatly increased. Additionally, peroxisomal isocitrate dehydrogenase was proven to be a main source of NADH for its reutilisation within these organelles (Corpas, et al., 1999). Interestingly, although the protein level and Vmax value of peroxisomal isoform remained unaltered in the young and senescent pea leaves, the substrate affinity of this enzyme was 11-fold increased during the natural senescence. A possible explanation to account for this phenomena is that the isocitrate dehydrogenase maintains the constant NADH level in order to eliminate the excess of ROS produced during aging (as the cofactor required for the reduction of oxidized glutathione) and therefore protects the peroxisomes against the oxidative stress. It is believed that other NADP-requiring isoforms of the enzyme play a similar role in the defence mechanisms of other cellular compartments (Moller and Rasmusson, 1998; Corpas, et al., 1999). Both the mitochondrial NAD-dependent (IDH) and cytosolic NADP-dependent (ICDH) isocitrate dehydrogenases were proposed to be responsible for the production of 2-oxoglutarate re-

quired by the nitrogen assimilation process catalysed by the glutamine synthetase / glutamate synthase (GS-GOGAT) cycle. Many previous experiments have attempted to elucidate the specific roles of both isoforms. Initially, it was the NAD-IDH that was believed to supply N metabolism with 2-OG, however there are many arguments in favour of the other isoform. They include higher citrate than 2-OG export rate to the cytosol (Hanning and Heldt, 1993; Hanning, et al., 1999) as well as the increase in ICDH transcript level in plants subjected to the N-limiting conditions (Scheible, et al., 1997b) or fed with nitrate (Fieuw, et al., 1995). Moreover, recent studies confirm the hypothesis of the cytosolic NADP-ICDH isoform being responsible for 2-oxoglutarate biosynthesis required for nitrate assimilation (Lemaitre and Hodges, 2006). In the contrary to these findings, previous experiments performed on antisense potato plants exhibiting strongly reduced ICDH activity (Kruse, et al., 1998) led the authors to the conclusion that ICDH isoform may be involved in the degradation of fatty acids or supply of NADH, particularly when the metabolic limitations of the oxidative pentose-phosphate pathway occur. Similar supporting role was assigned to the mitochondrial NADP-dependent enzyme (Gray, et al., 2004). Igamberdiev and Gardestroem (2003) proposed cytosolic NADP-ICDH to supply 2-oxoglutarate for photorespiratory ammonia refixation at limiting CO_2 conditions in the light. Recent studies additionally stress the role of this isoform in defensive response against variety of environmental stresses (Leterrier, et al., 2007). In summary, it is believed that the NAD-IDH isoform is responsible for maintaining the TCA cycle flux under steady-state conditions, the NADP-requiring enzyme supports the Krebs cycle and electron transfer chain with reducing equivalents in the conditions that disturb the balance between the carbon metabolism and electron transport.

4.2 Aim of work

Isocitrate dehydrogenases, which are located in multiple cellular compartments possess a vital function in central metabolism of plants. Although the various isoforms of isocitrate dehydrogenase have often been postulated to be engaged in the production of carbon skeletons and reducing equivalents for multiple biosynthetic reactions including the nitrogen assimilation process, their precise physiological role in plant metabolism remains unknown. For this reason, the aim of this work was to comprehensively evaluate the importance of mitochondrial (NAD-IDH) and cytosolic (NADP-ICDH) isocitrate dehydrogenases in metabolism of illuminated leaf. In order to achieve this I investigated the effect of deficient gene expression of the enzymes at the phenotypic, metabolic and physiological levels in tomato.

4.3 Results

4.3.1 Sequence analysis and generation of the NAD-IDH and NADP-ICDH transgenic plants

The tomato EST collection (Van der Hoeven, et al., 2002) contained seven tentative consensus (TC193092; TC164449; TC196623; TC198615; TC201555; TC202045; TC216549) encoding isocitrate dehydrogenase, however only three of them (TC193092; TC164449 and TC202045) showed high identity to the isocitrate dehydrogenases from other plant species. When the sequences of these genes were compared and visualized in a phylogenetic tree (Figure 32), two distinct clusters of enzymes were formed, based on the cofactor specificity. The first of them included TC193092 sequence, which was recognized as a NAD-dependent tomato IDH (*Sl*IDH1), encoding protein consisting of 393 amino acids. It shared 75% identity to NAD-IDH of tobacco (*Nicotiana tabacum*, CAA74776; Y14431) and 66% identity to IDH1 in Arabidopsis (*Arabidopsis thaliana*, NP_195252, At4g35260). The second cluster containing NADP-dependent isocitrate dehydrogenases included both TC164449 and TC202045 tomato genes. Assembly and sequence analysis of TC164449 revealed an open reading frame of 415 amino acids with high homologies to NADP-dependent ICDH (*Sl*ICDH2). Comparison with functionally characterized NADP-isocitrate dehydrogenases revealed 91% identity to tobacco (*Nicotiana tabacum*, P50218; X77944) and 90% identity to potato (*Solanum tuberosum*, CAA53300, X75638). The sequence analysis of TC202045 also revealed an open reading frame of 415 amino acids with high homologies to NADP-dependent ICDH (*Sl*ICDH1). Comparison of this isoform to functionally characterized NADP-dependent isocitrate dehydrogenases revealed 96% identity to tobacco (*Nicotiana tabacum*, P50218; X77944) and 98% identity to potato (*Solanum tuberosum*, P50217, X75638). *In silico* prediction indicates that *Sl*ICDH1 and 2 have a cytosolic location, whereas *Sl*IDH1 bears characteristics of a mitochondrial transit peptide sequence (data not shown).

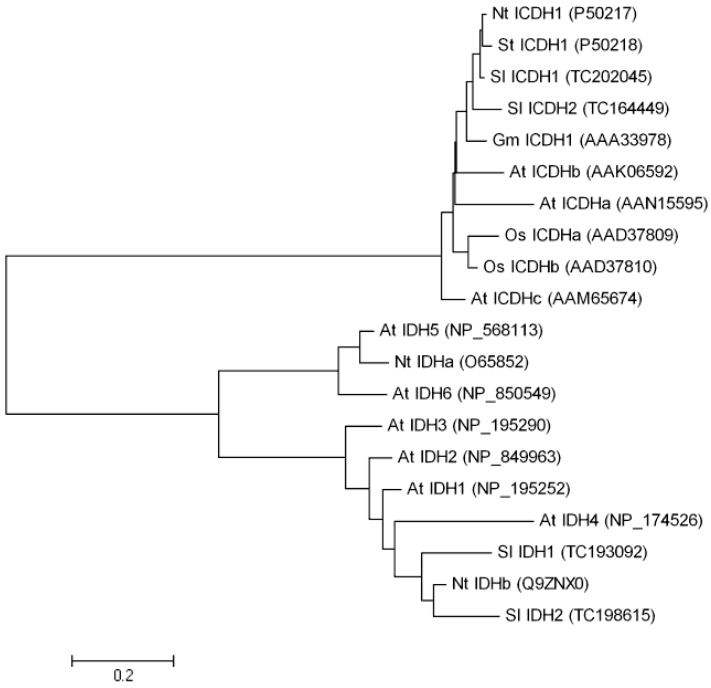


Figure 32: Dendrogram of *Sl*IDH and *Sl*ICDH amino acid sequences. The sequences were aligned using the ClustalW alignment program (Higgins and Sharp, 1988). Neighbor Joining tree (Saitou and Nei, 1987) was constructed with MEGA4 software (Tamura, et al., 2007). The protein accession numbers are given in brackets. The abbreviations used were: Nt, *Nicotiana tabacum*; St, *Solanum tuberosum*; Sl, *Solanum lycopersicum*; Gm, *Glycine max*; At, *Arabidopsis thaliana*; Os, Oryza sativa.

Investigation of the role of isocitrate dehydrogenase (IDH) in plant metabolism was performed by the analysis of transgenic tomato plants (*Solanum lycopersicum* cv. Moneymaker). The down regulation of the mitochondrial NAD-dependent isoform of IDH was achieved by myself by the introduction of a 734 bp fragment of the tomato gene *Sl*IDH1 (TC193092) in the antisense orientation into the Gateway transformation vector pK2WG7 (Karimi, et al., 2004). The transgenic NADP-ICDH plants were generated beforehand in Max-Planck Institute for Molecular Plant Physiology in Potsdam-Golm, Germany by introduction of a 527 bp fragment of *Sl*ICDH1 tomato gene (TC202045) into a RNAi transformation vector pK7GWIWG2(I) in both antisense and sense orientation, separated by 643 bp intron (Karimi, et al., 2004). In both cases the gene of interest was inserted between the CaMV 35S promoter and the T35S terminator ensuring highly effective and constant transcription. Following *Agrobacterium tumefaciens* – mediated transformation, the resulting two transgenic plant sets were subsequently grown in the greenhouse conditions and screened by quantification of transcript, protein and enzymatic activity level (data not shown). Finally, I selected two lines of NAD-IDH that revealed limited NAD-IDH activity and transcript level (Figure 33). Additionally, I was given three lines of NADP-ICDH genotypes that were significantly limited in the transcript level of cytosolic NADP-ICDH and total NADP-

isocitrate dehydrogenase activity (Figure 34). These plants were clonally propagated and transferred to the greenhouse for detailed physiological analysis.

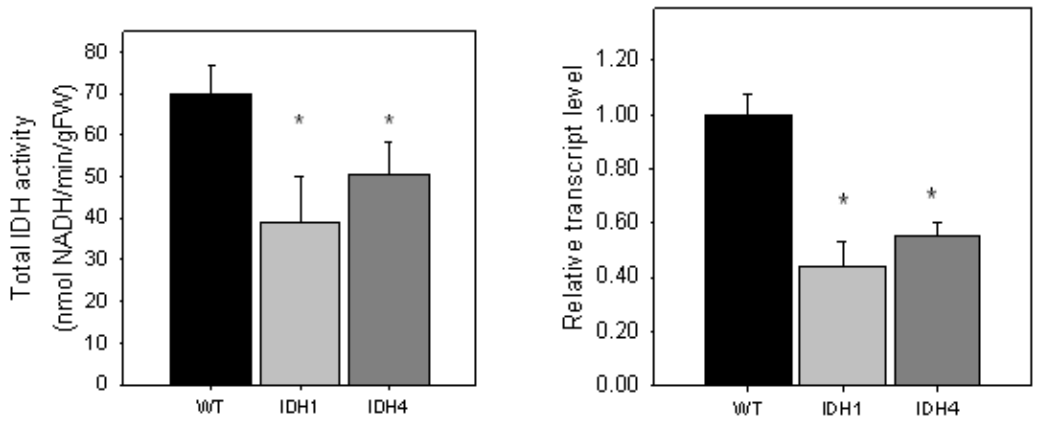


Figure 33: Enzymatic activity of mitochondrial NAD-IDH (on the left hand side) and RT-PCR-based transcript level of *Sl*IDH1 tomato gene (on the right hand side) in the leaves of selected NAD-IDH transgenic lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

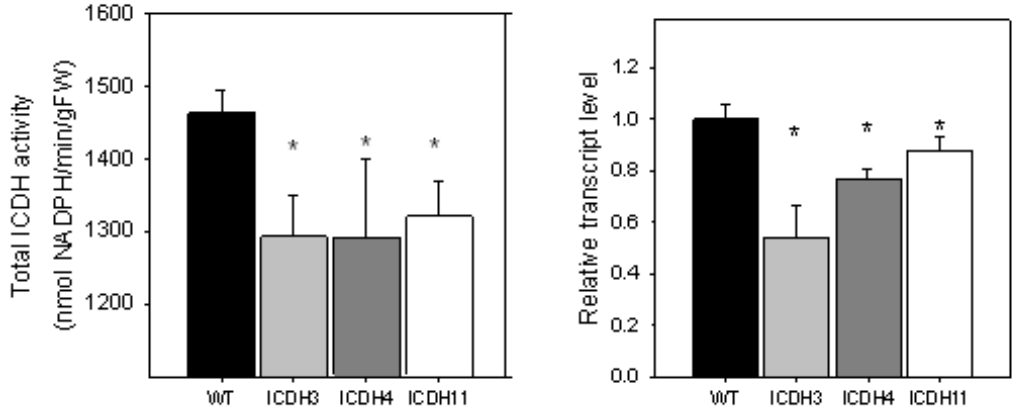


Figure 34: Enzymatic activity of total NADP-ICDH (on the left hand side) and RT-PCR-based transcript level of cytosolic *Sl*ICDH1 tomato gene (on the right hand side) in the leaves of selected NADP-ICDH transgenic lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

4.3.2 Phenotypic characterization of the NAD-IDH and NADP-ICDH transgenic plants

Interesting information was revealed when the phenotypic traits were analyzed in both sets of the transgenic plants grown in the greenhouse conditions until full maturity. The lines characterized by diminished activity of mitochondrial isoform of IDH exhibited only a minor decrease in root dry mass and no significant changes in fresh weight (data not shown) and dry weight of other organs (Figure 35, upper graphs), with the exception of limited fruit weight. The transgenic plants displayed relatively unaltered total biomass accumulation and height comparable to wild type plants (Figure 37). The evaluation of fruit yield of NAD-IDH anti-sense plants revealed a decreasing tendency in total fresh weight (data not shown) and dry weight (Figure 36, upper graphs), in addition to significantly limited mean fresh weight of the transgenic fruits. Moreover, the diameter of the NAD-IDH fruits was markedly decreased in both transgenic lines, as compared to wild type plants. Nevertheless, the number of flowers and fruits remained unaltered and the time of flowering was synchronic to that of the wild type plants (Figure 36 and Figure 38).

Interestingly, highly similar results were obtained for NADP-ICDH tomatoes (Figure 35, Figure 36 lower graphs). The fruits of the transgenic lines were characterized by lower fresh and dry weight, diminished mean fresh weight and smaller diameter; however the number of fruits was markedly increased. Although the NADP-ICDH plants generated many small fruits, they exhibited only minor alterations of vegetative growth. Whilst the total dry weight of leaves, stems and whole plants remained unaltered, the root formation was significantly increased in all lines with diminished NADP-ICDH activity.

This study showed that the reduction in the performance of either mitochondrial or cytosolic isocitrate dehydrogenase influences root growth and in both cases, it had strong negative impact on fruit production in tomato.

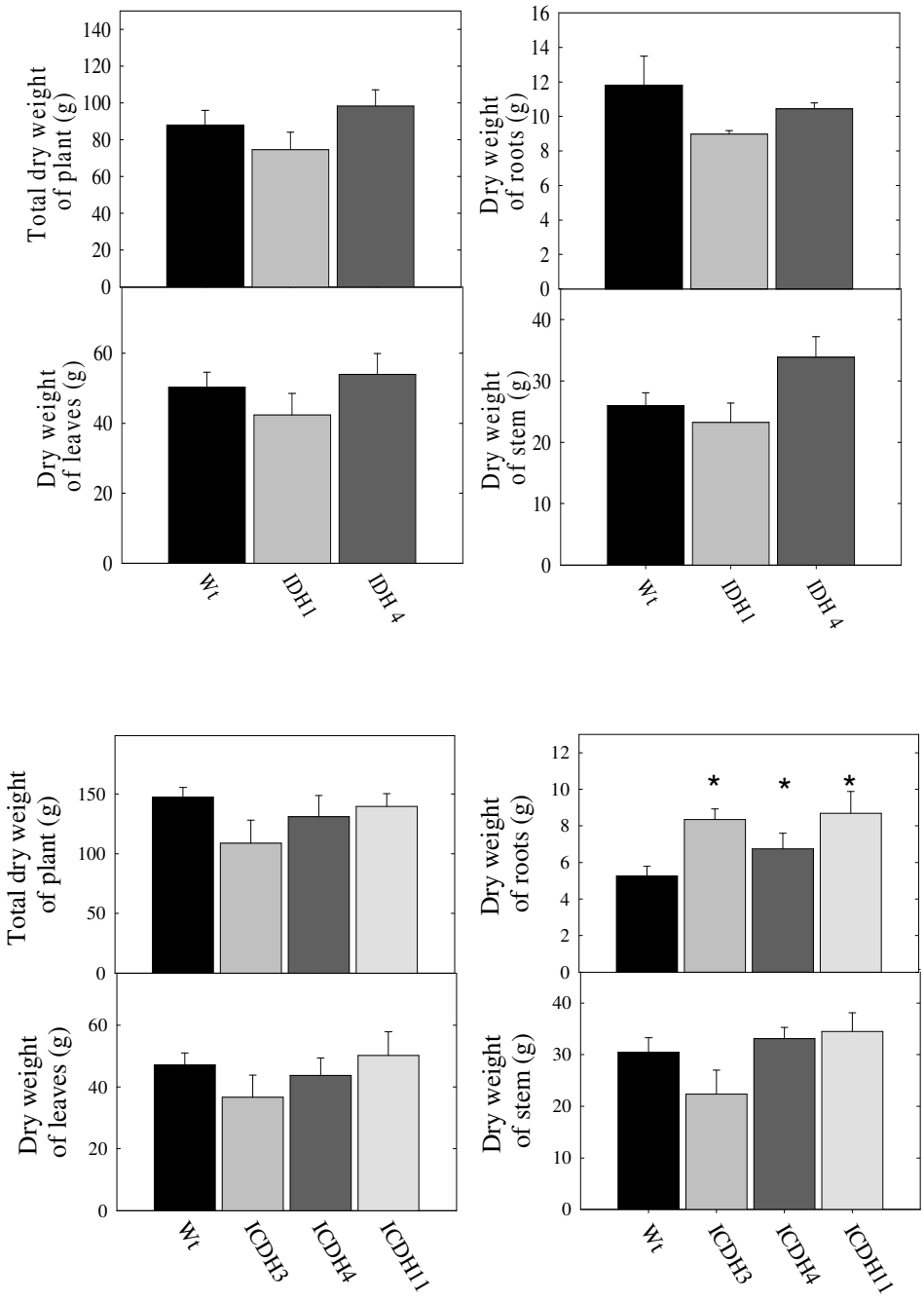


Figure 35: Dry weight of different plant organs of eleven week old NAD-IDH and NADP-ICDH transgenic and wild type plants growing in greenhouse conditions. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

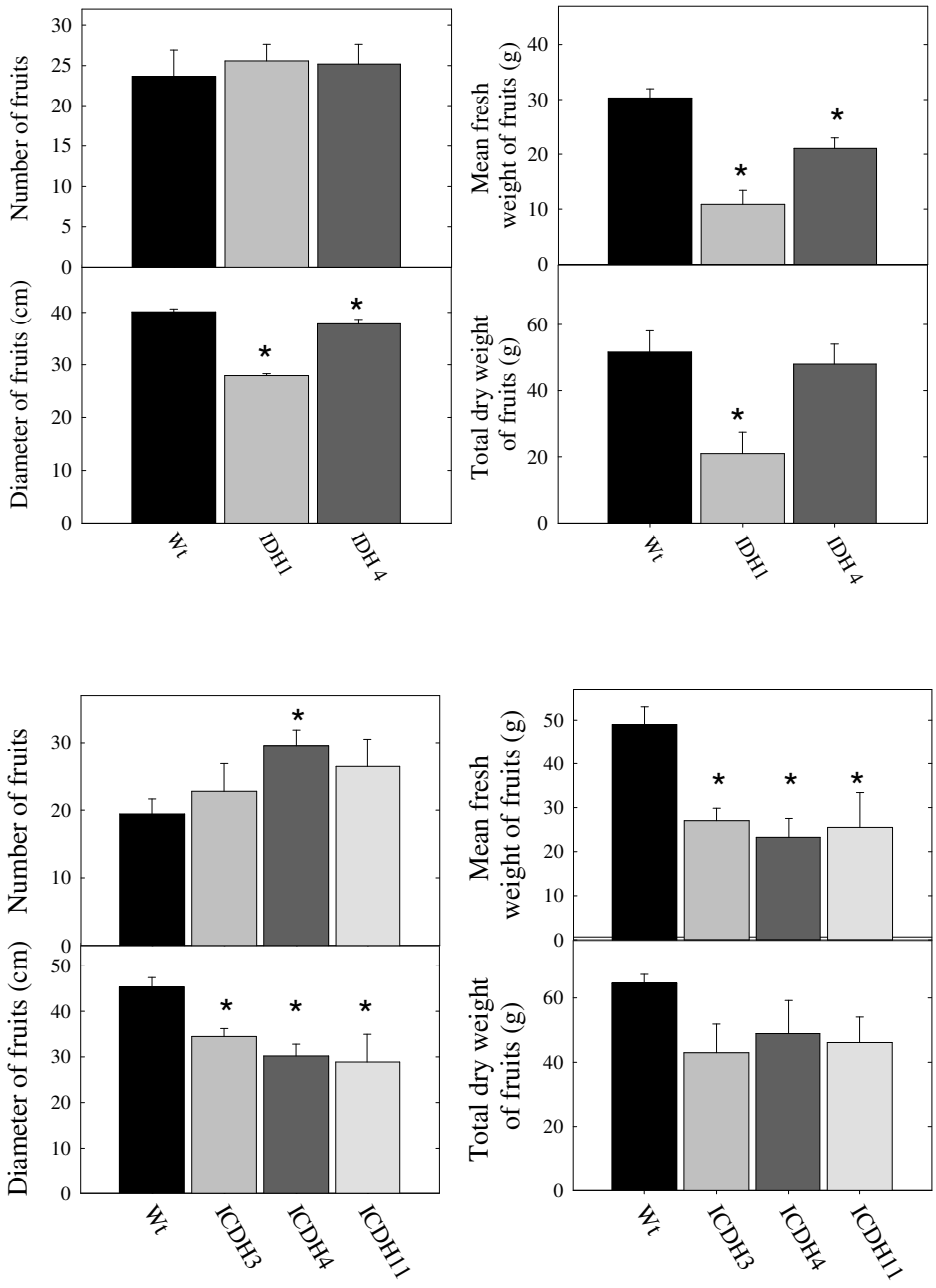


Figure 36: Fruit performance of eleven week old NAD-IDH and NADP-ICDH transgenic and wild type plants growing in greenhouse conditions. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

Figure 37: Photograph of five week old NAD-IDH transgenics and wild type plant growing in the greenhouse conditions. From the left hand side: WT, line IDH1, line IDH4.

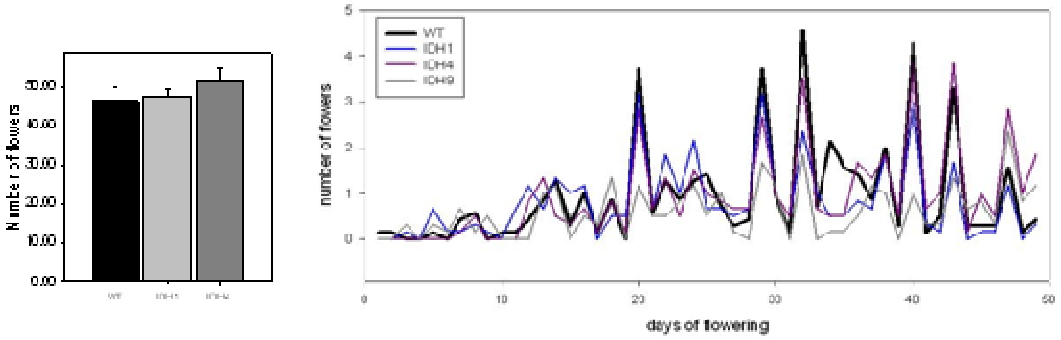


Figure 38: Total number of flowers and time of flowering of NAD-IDH transgenics and wild type plants under the greenhouse conditions. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

4.3.3 Evaluation of the photosynthetic and respiratory performance of the NAD-IDH and NADP-ICDH transgenic plants

Following the analysis of morphological aspects of the transgenic lines, I next studied the physiological alterations that resulted from decreased isocitrate dehydrogenase activity. Surprisingly, neither of the two transgenic genotypes revealed any major changes in the leaf photosynthetic performance. Assimilation (Figure 39) and transpiration rates as well as stomatal conductance (data not shown) of both NAD-IDH and NADP-ICDH transgenic leaves resembled those of the wild types. Similarly, the rate of chloroplastic electron transfer, quantified by the use of PAM fluorometer was found to be unaltered in both transgenic genotypes (data not shown). Nevertheless, when I evaluated chlorophyll fluorescence parameters, a clear reduction in the maximum efficiency of PSII (Fv/Fm) was observed in both NAD-IDH and NADP-ICDH lines, following dark adaptation (Figure 40).

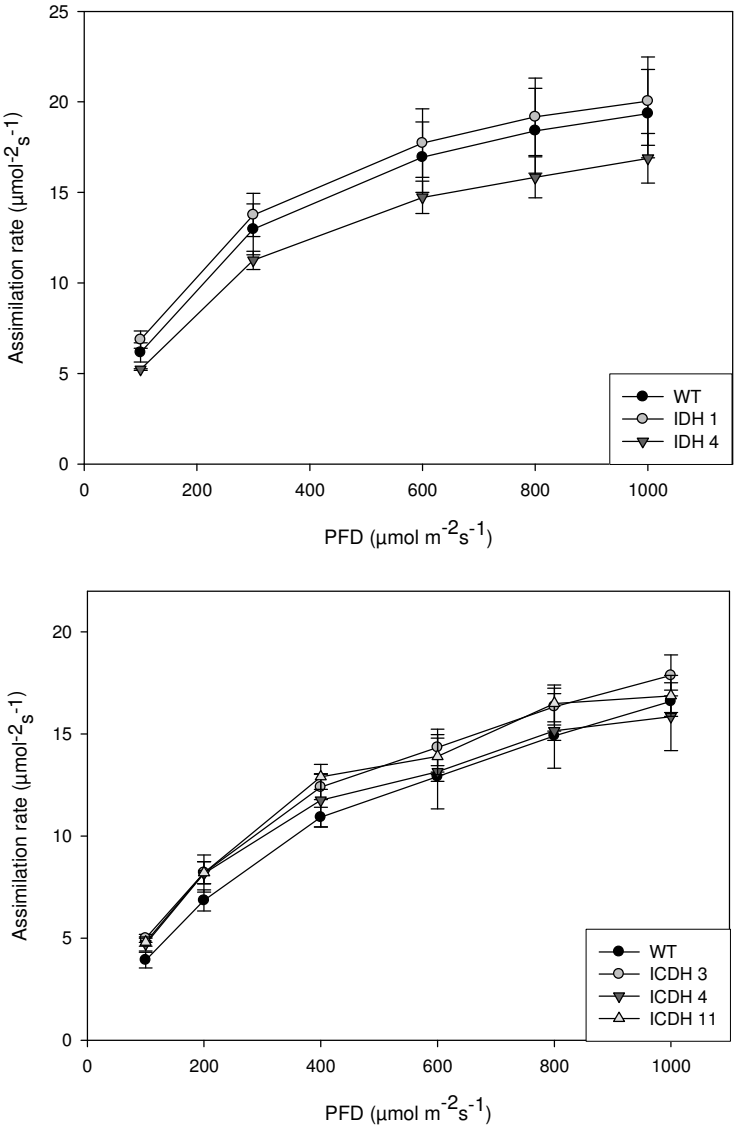


Figure 39: Assimilation rate of illuminated leaves of four week old NAD-IDH and NADP-ICDH transgenic lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line.

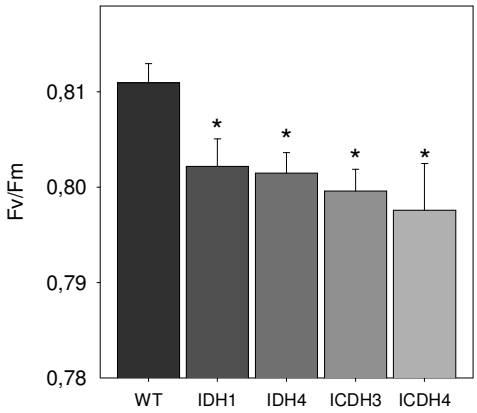


Figure 40: Estimated maximum photosynthetic (PSII) efficiency in dark-adapted four week old NAD-IDH and NADP-ICDH transgenic plants. The data is presented as Fv/Fm ratio measured by the use of Licor-6400 gas-exchange system. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p< 0.05$) values in comparison to wild type as determined by the *t* test.

The performance of photosynthetic assimilation in the transgenic plants was subsequently quantified in a feeding experiment in which illuminated leaf discs were incubated in $^{14}CO_2$ - rich atmosphere in an oxygen electrode for thirty minutes (Figure 41). The uptake and redistribution of radiolabel for NAD-IDH and NADP-ICDH plants were comparable to those observed for control plants. That said, the data essentially confirm my previous observation that the decreased activity of either mitochondrial or cytosolic isoform of isocitrate dehydrogenase has rather minor influence on plant photosynthetic ability, although the inhibition of either of these enzymes results in a considerable reduction in the maximum efficiency of PSII.

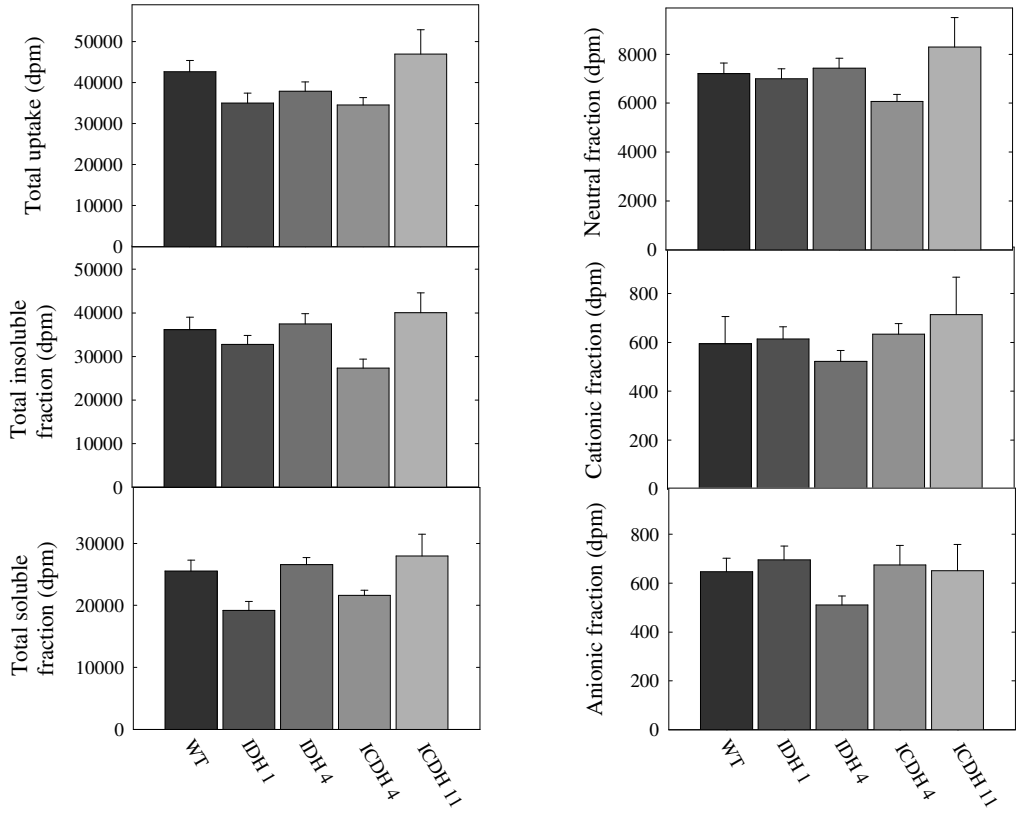


Figure 41: Photosynthetic assimilation and partitioning of carbon in six week old NAD-IDH and NADP-ICDH lines transgenic and wild type plants (in black). The mean enrichment ± SE of the radioactive carbon into fractions of leaf discs fed with $^{14}CO_2$ at the onset of illumination in the oxygen electrode was quantified on the basis of five individual plants per line. An asterisk indicates significantly different ($p< 0.05$) values in comparison to wild type as determined by the *t* test.

Having investigated the yield of photosynthesis, I next analyzed whether the down regulation of mitochondrial isoform of IDH influences plant respiratory performance. In order to distinguish possible alterations of the main carbohydrate oxidation pathways in the transgenics I analysed $^{14}CO_2$ evolution from leaf discs treated with [1-^{14}C], [2-^{14}C], [3:4-^{14}C], or [6-^{14}C] glucose in the light over a period of six hours. As shown by ap Rees and Beevers (1960) carbon dioxide can be released from the C1 position by the action of enzymes that are not associated with mitochondrial respiration, in contrast to carbon dioxide evolution from the C3:4 positions of glucose . Therefore, the ratio of $^{14}CO_2$ evolution from C1 to C3:4 positions of glucose provides an indication of the relative rate of the TCA cycle with respect to other processes of carbohydrate oxidation. The experiment revealed that carbon dioxide release from C1-labelled glucose was far in excess in both NAD-IDH transgenic lines to that observed in wild type plants (Figure 42). Moreover, the $^{14}CO_2$ evolution from the C3:4 position was much lower in the transgenic line

IDH1 than in the wild type. That said, these lines exhibited a significantly elevated rate of CO_2 evolution, as assessed by the ratio C1/C3:4. This observation suggests that leaves of the transgenic plants may display a reduced ability of the TCA cycle to perform carbohydrate oxidation.

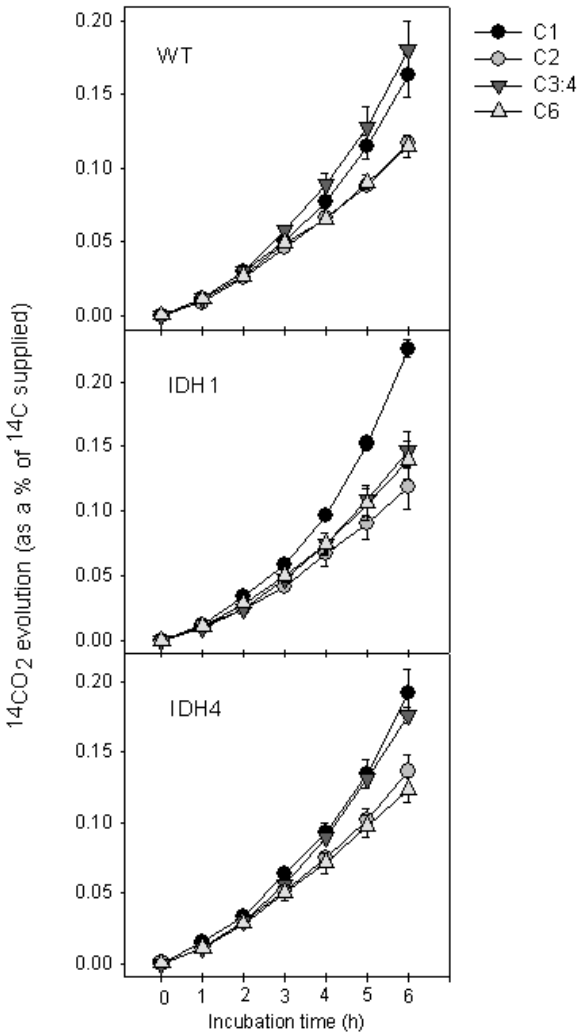
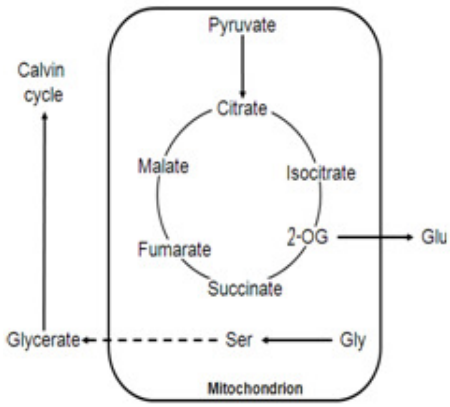


Figure 42: Respiratory performance of five-week old NAD-IDH transgenic and wild type plants presented as $^{14}CO_2$ evolution from the illuminated leaf discs fed with 10 mM MES-KOH solution, pH 6.5, 0.3 mM glucose supplemented with 2.32 kBq mL^{-1} of [1-^{14}C]-, [2-^{14}C]-, [3:4-^{14}C]-, or [6-^{14}C] glucose at an irradiance of 200 μmol m^{-2} s^{-1} for six hours. The released radiolabel was captured in hourly intervals in 10% KOH trap and subsequently quantified by liquid scintillation counting. Values are presented as mean ± SE of determination on six individual plants per line.

The second approach towards an assessment of the respiration performance in NAD-IDH transgenic plants included an evaluation of the relative isotope redistribution within the transgenic and wild type leaves. The ^{13}C-labeled pyruvate was supplied to the leaf via transpiration stream for a period of three hours in the

light. Subsequently, GC-MS approach was used to evaluate the redistribution of ^{13}C to TCA cycle, GABA shunt and photorespiratory pathway (Figure 43). These studies indicated that the isotope supply to citrate remained unaltered in the transgenic leaves, however the carbon flow towards the remaining TCA cycle intermediates was massively reduced. The flux in the direction towards isocitrate and 2-oxoglutarate as well as fumarate and malate was significantly decreased in all transgenic lines. Similarly, a limited label redistribution from 2-oxoglutarate towards glutamate was clearly observed in IDH4 line. However, the carbon supply towards succinate and GABA, a metabolite involved in TCA cycle bypass pathway (Studart-Guimaraes, et al., 2007) was only marginally, if any inhibited. In contrast to that general decrease in isotope redistribution to the TCA cycle intermediates, a clearly increased carbon flux was observed towards glycine (significantly in line IDH4) and serine (line IDH1), vital intermediates of plant photorespiratory pathway.



	WT			IDH1			IDH4		
	µmol C_1 equivalents $g^{-1}FW\ h^{-1}$								
Citrate	1,031	±	0,110	0,921	±	0,051	0,896	±	0,137
Isocitrate	0,163	±	0,013	**0,119**	±	**0,004**	**0,124**	±	**0,012**
2-OG	0,111	±	0,028	**0,035**	±	**0,001**	**0,038**	±	**0,004**
Succinate	5,129	±	0,846	3,551	±	0,204	3,866	±	0,357
Fumarate	0,039	±	0,003	**0,024**	±	**0,004**	**0,025**	±	**0,005**
Malate	7,865	±	0,976	**5,173**	±	**0,179**	**5,237**	±	**0,577**
Glutamate	3,425	±	0,589	2,448	±	0,107	**1,630**	±	**0,217**
Glycine	0,040	±	0,009	0,063	±	0,006	**0,090**	±	**0,017**
Serine	0,088	±	0,025	**0,169**	±	**0,018**	0,158	±	0,036
Glycerate	0,032	±	0,004	0,030	±	0,001	0,032	±	0,003
GABA	4,066	±	0,612	3,906	±	0,241	3,216	±	0,382

Figure 43: Redistribution of radiolabel following feeding of 20mM [U-^{13}C] sodium pyruvate to the leaves excised from six-week old NAD-IDH transgenic and wild-type plants via the petioles. The experiment was performed for three hours under high light conditions in the phytotron. The scheme on the left hand side is provided to visualize the distribution of [U-^{13}C] pyruvate within central metabolism of plants. The table on the right hand side contains data presented as the accumulation of micromolar C1 equivalents per hour per gram of leaf fresh weight. Values are calculated as mean ± SE of determination on six individual plants per line. Bold font indicates significantly different ($p< 0.05$) values in comparison to wild type as determined by the *t* test.

In summary, the data obtained from two independent respiration measurements described above, hint towards a restriction in pyruvate-derived fluxes and an elevation in the activity of mitochondrially-located photorespiratory enzymes in the leaves of NAD-IDH antisense plants.

4.3.4 Metabolic profiling of the NAD-IDH and NADP-ICDH transgenic plants

4.3.4.1 Leaf carbohydrate content

Next, I decided to evaluate the metabolic content of the transgenic leaves to assess whether the decreased enzymatic activity of isocitrate dehydrogenase caused any modifications in central and secondary metabolism of tomato. The level of hexoses across the diurnal cycle was unchanged in NAD-IDH lines and showed only minor decreasing tendency in the NADP-ICDH plants, as compared to control plants (Figure 44). In contrast, the starch content was significantly decreased in both sets of transgenic genotypes.

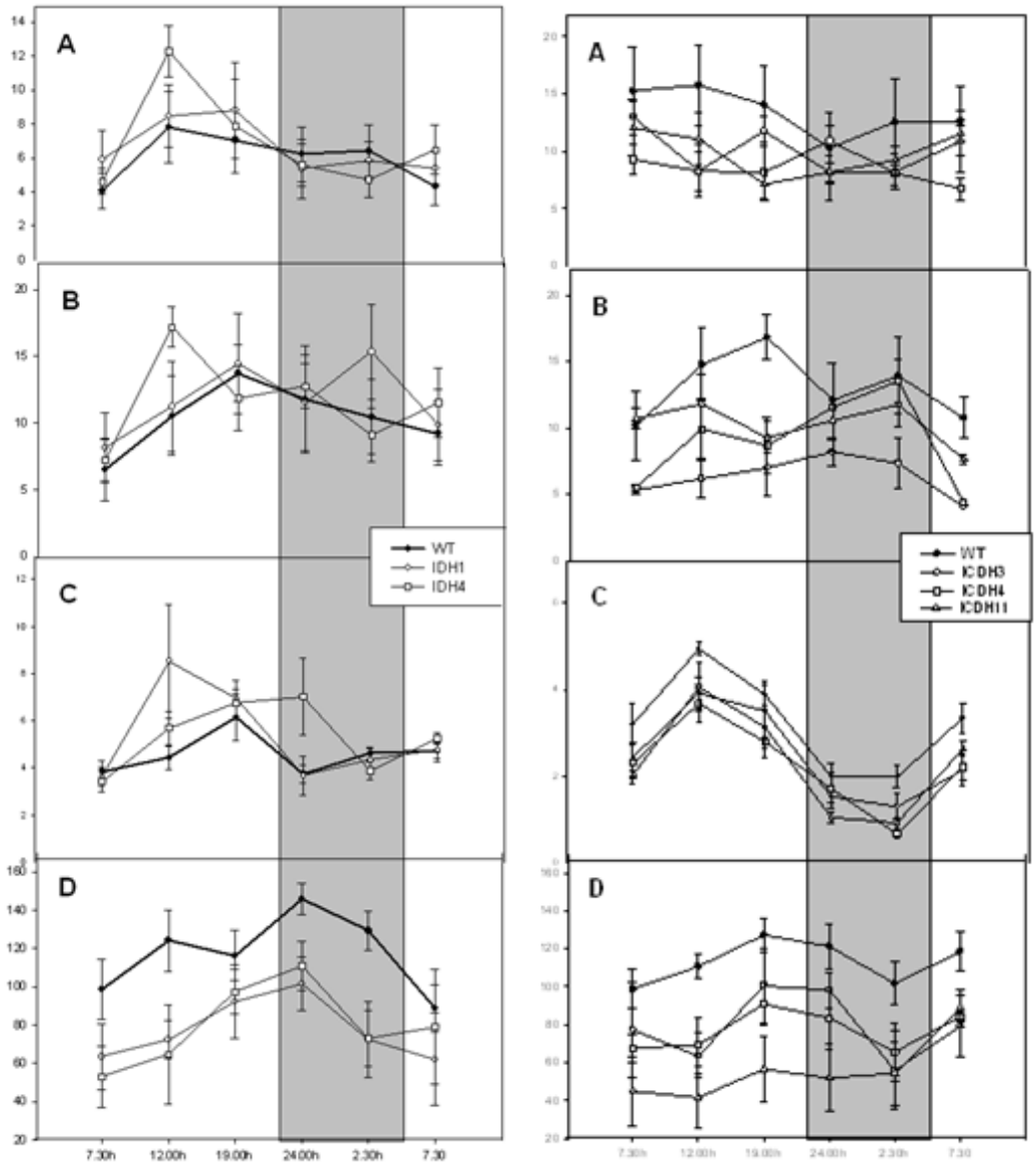


Figure 44: Diurnal changes in carbohydrate content in the source leaves of six week old NAD-IDH (on the left hand side) and NADP-ICDH (on the right hand side) transgenic and control plants. The gray bar indicates the dark period. Values are presented as mean ± SE of determination on six individual plants per line. Glucose (A), fructose (B), sucrose (C) and starch (D) are presented as a micromol of glucose per gram of fresh weight (µmol glc gFW^{-1}).

4.3.4.2 Leaf nucleotide level

The leaf nucleotide content of NAD-IDH antisense plants, as analyzed by high-performance liquid chromatography (HPLC) technique was unaltered in comparison to the wild type control (data not shown), however the NADP-ICDH lines revealed some minor alterations in the level of diphosphonucleotides (Table 6).

Although the ADP content was significantly decreased in two lines ICDH4 and ICDH11, this change was not accompanied by consistent changes in the ATP/ADP ratio. Furthermore, no major alterations in the levels of guanosine- and cytidine-phosphates were observed in the transgenic leaves (data not shown).

Table 6: HPLC-based quantification of nucleotides content in the source leaves of five week old NADP-ICDH transgenic lines and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. The bold font was used to indicate significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

Nucleotides level (nmol g FW $^{-1}$)

	WT	ICDH3	ICDH4	ICDH11
ADP-Glc	5.5 ± 0.7	7.5 ± 1.9	4.5 ± 1.1	6.1 ± 1.1
ATP	30.9 ± 8.2	18.5 ± 4.1	23.9 ± 4.4	35.8 ± 6.1
ADP	15.8 ± 0.6	15.0 ± 1.5	**12.8 ± 0.8**	**13.3 ± 0.8**
ATP/ADP	2.0 ± 0.2	1.2 ± 0.3	1.9 ± 0.4	2.7 ± 0.4
∑ adenylates	52.1 ± 8.3	41.0 ± 4.8	41.3 ± 4.6	55.1 ± 6.2
UDP-Glc	87.7 ± 8.3	**53.8 ± 5.3**	73.7 ± 4.1	81.1 ± 4.8
UTP	22.9 ± 4.8	15.5 ± 3.1	25.2 ± 3.2	24.9 ± 3.6
UDP	4.7 ± 0.9	2.0 ± 1.0	**9.4 ± 1.6**	4.4 ± 1.0
UTP/UDP	4.9 ± 1.0	7.7 ± 2.3	2.7 ± 0.8	5.7 ± 1.5
∑ uridinylates	115.3 ± 9.6	**71.3 ± 6.3**	108.4 ± 5.4	110.5 ± 6.0

During the decarboxylation of isocitrate to 2-oxoglutarate catalyzed by both IDH and ICDH the reduced coenzyme NAD(P)H is being produced. It might therefore be expected that reduction in the activity of these enzymes effects the redox balance in the transformants. I therefore decided to assay the levels of pyridines in the leaves of five week old wild type and transgenic plants. Interestingly, the reduced products of isocitrate dehydrogenase enzymatic activity, namely NADH and NADPH were found to be significantly reduced in all transgenic genotypes (Figure 45). Nevertheless, the NAD-IDH lines were characterized by unaltered level of NAD and NADP and the calculated NADH/NAD and NADPH/NADP ratios were surprisingly comparable to those of wild type. In contrary to these findings, the NADP-ICDH plants showed increased level of NAD, which resulted in lowered NADH/NAD ratio, significant for all three lines ($p<0.01$). Although, these plants presented both lowered NADPH and NADP level when compared to wild type controls, the calculated NADPH/NADP ratio remained unaltered.

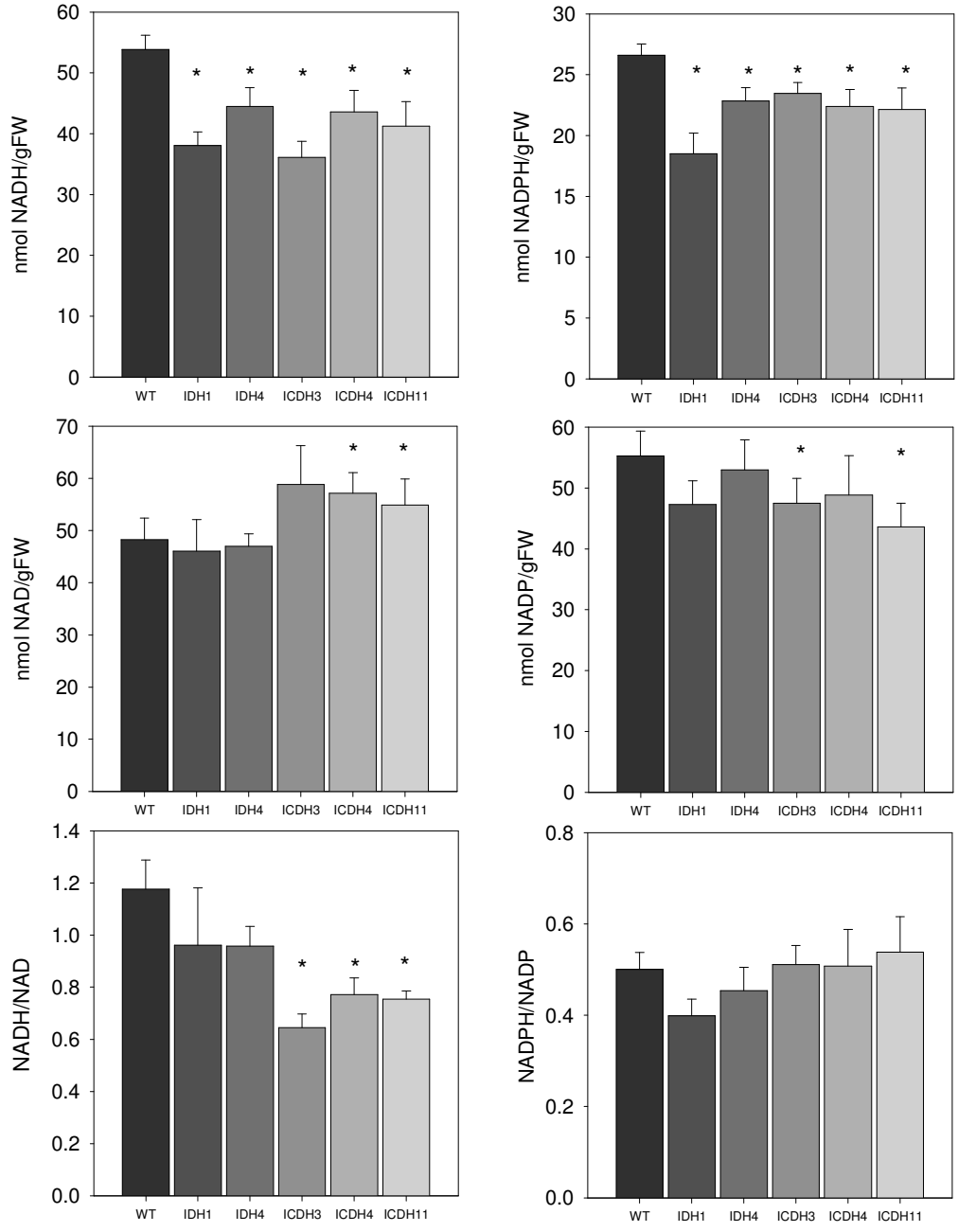


Figure 45: Pyridine nucleotide levels and ratios in leaves of NAD-IDH and NADP-ICDH transgenic and control plants. The leaf material was harvested in the middle of the light period, from five week old plants. Values are presented as mean ± SE of determination on six individual plants per line. Asterisk was used to indicate significantly different ($p < 0.05$) values in comparison to wild type as determined by the *t* test.

4.3.4.3 GC-MS profiling of primary metabolites in the NAD-IDH and NADP-ICDH transgenic leaves

Detailed information concerning leaf metabolic perturbations following the genetic modification of isocitrate dehydrogenase activity was obtained by the use of metabolic profiling based on the powerful GC-MS technique. This study revealed considerable differences in the level of primary metabolites between the both sets of transgenic and wild type plants (Table 7). The NAD-IDH tomato plants were characterized by several notable changes in the amino acid content, including significantly reduced level of β-alanine and proline in all lines and glycine in IDH1 line, in addition to significantly increased asparagine and tyramine in IDH1 line. Among organic acid content, a clear reduction of the TCA cycle intermediates, such as malate and succinate in IDH1 line and pyruvate in IDH4 line was noticed. However, this tendency was not present for 2-oxoglutarate and isocitrate, which content remained unchanged in the NAD-IDH transformants. This phenomenon was not particularly surprising, given the presence of multiple enzymatic isoforms capable of catalyzing conversion of these metabolites. Several other interesting changes were also observed such as the significant reduction in threonate and maleate in IDH1 line; glycerate and citramalate in all lines and GABA in IDH4, as well as the increase in saccharic acid in IDH1 line.

Generally, the metabolite profile of NADP-ICDH transgenic plants was found to be very distinct to that of NAD-IDH plants. The significant elevation of alanine and phenylalanine in all lines was accompanied by an accumulation of metabolites directly involved in nitrogen assimilation process, such as asparagine, glutamine, glutamate and GABA. Two amino acids, namely proline and tyramine showed consistent decrease among the transgenic lines. Additionally, serine and glycine were significantly decreased in ICDH3 line. The TCA cycle intermediates, such as citrate, isocitrate, 2-OG, succinate and fumarate remained at the wild type level in all transgenic lines, hinting that the performance of this pathway was not altered by the decrease in NADP-ICDH activity. Moreover, consistent accumulation of maleate was observed in all transgenic lines, whereas the level of glycolate (ICDH3 line) and glycerate (two lines) was significantly limited. Interestingly, NADP-ICDH leaves were characterized by general decreasing tendency in the content of carbohydrates, that became significant for glucose and fructose in two transgenic lines. Furthermore, sucrose content was limited in ICDH3 line and maltose in ICDH11 line. This trend was well manifested in the level of glucose- and fructose-6-phosphates, that was two fold decreased in ICDH3 and ICDH4 lines. In contrast to these findings, NAD-IDH plants did not reveal any major changes in sugar content. Similarly, the latter plants were invariant in the level of fatty acids, whereas NADP-ICDH transgenics accumulated both palmitic and stearic acids. The level of secondary metabolites that were quantified in leaves, such as nicotinate, caffeate, dehydroascorbate and dopamine was unaltered in both NAD-IDH and NADP-ICDH transgenic plants (data not shown).

Table 7: Relative metabolite content of NAD-IDH and NADP-ICDH plants obtained by the use of GC-MS technique. Fully expanded leaves were collected from the greenhouse-grown plants at the age of five weeks. Data were normalised to the mean response calculated for wild type control. Values are presented as mean ± SE of determination on six individual plants per line. Green and red colours indicate respectively values that decrease and increase significantly ($p < 0.05$) in relation to the wild type, as determined by the *t* test.

	WT	IDH1	IDH4	ICDH3	ICDH4	ICDH11
Beta-alanine	1.000 ± 0.027	0.818 ± 0.062	0.698 0.105	1,184 ± 0,079	1,135 ± 0,219	0,934 ± 0,088
Alanine	1.000 ± 0.170	1.233 ± 0.320	0.757 ± 0.121	1,411 ± 0,207	2,145 ± 0,179	1,848 ± 0,050
Arginine	1.000 ± 0.096	0.971 ± 0.021	0.918 ± 0.100	0,589 ± 0,135	1,062 ± 0,079	1,017 ± 0,057
Asparagine	1.000 ± 0.047	1.513 ± 0.116	0.881 ± 0.075	1,244 ± 0,074	1,893 ± 0,384	1,519 ± 0,081
Aspartate	1.000 ± 0.048	0.941 ± 0.060	0.908 ± 0.112	1,012 ± 0,326	1,662 ± 0,355	0,857 ± 0,253
GABA	1.000 ± 0.036	0.924 ± 0.064	0.832 ± 0.055	1,712 ± 0,207	1,511 ± 0,290	1,692 ± 0,197
Glutamate	1.000 ± 0.024	0.914 ± 0.045	0.919 ± 0.049	1,709 ± 0,307	1,593 ± 0,303	1,101 ± 0,142
Glutamine	1.000 ± 0.152	0.849 ± 0.103	0.725 ± 0.119	1,773 ± 0,334	2,245 ± 0,279	2,020 ± 0,310
Glycine	1.000 ± 0.063	0.770 ± 0.026	1.153 ± 0.064	0,309 ± 0,293	1,096 ± 0,294	1,068 ± 0,233
Isoleucine	1.000 ± 0.069	1.026 ± 0.084	1.039 ± 0.037	1,154 ± 0,290	1,067 ± 0,270	1,284 ± 0,141
Phenylalanine	1.000 ± 0.051	0.855 ± 0.047	1.069 ± 0.074	1,868 ± 0,240	1,306 ± 0,226	1,731 ± 0,189
Proline	1.000 ± 0.065	0.630 ± 0.047	0.397 ± 0.084	0,230 ± 0,032	0,533 ± 0,070	0,798 ± 0,059
Serine	1.000 ± 0.045	1.064 ± 0.046	1.058 ± 0.032	0,553 ± 0,206	1,152 ± 0,235	0,863 ± 0,249
Tryptophan	1.000 ± 0.087	0.823 ± 0.107	1.021 ± 0.090	1,361 ± 0,185	0,958 ± 0,343	1,152 ± 0,245
Tyramine	1.000 ± 0.083	1.477 ± 0.168	1.110 ± 0.080	0,792 ± 0,130	0,661 ± 0,100	0,670 ± 0,150
Valine	1.000 ± 0.054	0.955 ± 0.106	1.047 ± 0.044	1,298 ± 0,231	1,097 ± 0,254	1,389 ± 0,125
Citrate	1.000 ± 0.059	1.081 ± 0.121	1.056 ± 0.032	1,232 ± 0,087	1,198 ± 0,085	1,155 ± 0,265
Fumarate	1.000 ± 0.065	0.871 ± 0.097	1.107 ± 0.145	0,783 ± 0,074	0,953 ± 0,140	0,741 ± 0,269
2-OG	1.000 ± 0.098	1.211 ± 0.132	1.047 ± 0.164	1,122 ± 0,121	0,889 ± 0,311	1,287 ± 0,315
Glycerate	1.000 ± 0.063	0.620 ± 0.122	0.803 ± 0.047	0,371 ± 0,133	0,488 ± 0,111	1,060 ± 0,188
Glycolate	1.000 ± 0.044	0.813 ± 0.061	1.018 ± 0.047	0,782 ± 0,276	0,958 ± 0,235	1,061 ± 0,233
Isocitrate	1.000 ± 0.051	1.138 ± 0.094	0.915 ± 0.037	1,182 ± 0,151	0,894 ± 0,254	1,256 ± 0,237
Maleate	1.000 ± 0.074	0.568 ± 0.083	0.995 ± 0.083	3,162 ± 0,249	3,538 ± 0,350	1,491 ± 0,226
Citramalate	1.000 ± 0.046	0.613 ± 0.052	0.884 ± 0.049	0,599 ± 0,274	0,850 ± 0,240	1.03 ± 0.29
Malate	1.000 ± 0.022	0.888 ± 0.026	1.004 ± 0.014	1,385 ± 0,087	0,974 ± 0,109	0,964 ± 0,150
Pyruvate	1.000 ± 0.067	0.980 ± 0.131	0.740 ± 0.077	1,015 ± 0,151	1,096 ± 0,119	1,364 ± 0,162
Saccharate	1.000 ± 0.091	1.557 ± 0.208	1.149 ± 0.137	0,763 ± 0,209	1,109 ± 0,408	1,419 ± 0,279
Succinate	1.000 ± 0.080	0.701 ± 0.073	0.880 ± 0.149	0,866 ± 0,165	1,052 ± 0,084	1,012 ± 0,152
Threonate	1.000 ± 0.087	0.568 ± 0.070	0.859 ± 0.208	0,599 ± 0,141	1,148 ± 0,074	1,111 ± 0,160
Fructose	1.000 ± 0.036	0.945 ± 0.011	0.952 ± 0.042	0,769 ± 0,032	0,494 ± 0,162	0,934 ± 0,126
Glucose	1.000 ± 0.029	1.006 ± 0.026	1.035 ± 0.034	0,922 ± 0,205	0,404 ± 0,131	0,749 ± 0,065
Maltose	1.000 ± 0.047	0.771 ± 0.091	0.751 ± 0.071	0,889 ± 0,153	0,899 ± 0,099	0,562 ± 0,354
Sucrose	1.000 ± 0.035	0.974 ± 0.019	0.954 ± 0.031	0,762 ± 0,104	0,883 ± 0,105	1,054 ± 0,098
Trehalose	1.000 ± 0.045	0.856 ± 0.058	0.959 ± 0.058	0,812 ± 0,065	0,872 ± 0,059	1,138 ± 0,139
Glu-6-P	1.000 ± 0,091	1,304 ± 0,313	0,923 ± 0,110	0,559 ± 0,166	0,527 ± 0,445	0,826 ± 0,248
Fru-6-P	1.000 ± 0,170	1,196 ± 0,191	0,855 ± 0,208	0,495 ± 0,206	0,539 ± 0,243	0,809 ± 0,279
Palmitic acid	1.000 ± 0,050	1,060 ± 0,059	0,897 ± 0,060	1,570 ± 0,041	2,020 ± 0,047	1,970 ± 0,066
Stearic acid	1.000 ± 0,029	1,053 ± 0,031	1,081 ± 0,036	2,230 ± 0,037	2,500 ± 0,054	1,360 ± 0,061

4.3.4.4 Nitrate, total amino acid and protein level

Given that both mitochondrial and cytosolic isoforms of isocitrate dehydrogenase were proposed to be indirectly engaged in nitrogen assimilation process, I spectrophotometrically assayed nitrate as well as total amino acids and total insoluble protein in leaves (Figure 46). As it perhaps could be expected the intracellular nitrate content was significantly increased in the leaves of all transgenic genotypes, when compared to wild type plants. The NAD-IDH plants were characterized by unaltered total amino acid level but largely elevated protein level, whereas NADP-ICDH transgenics showed no changes in leaf total amino acid and protein content.

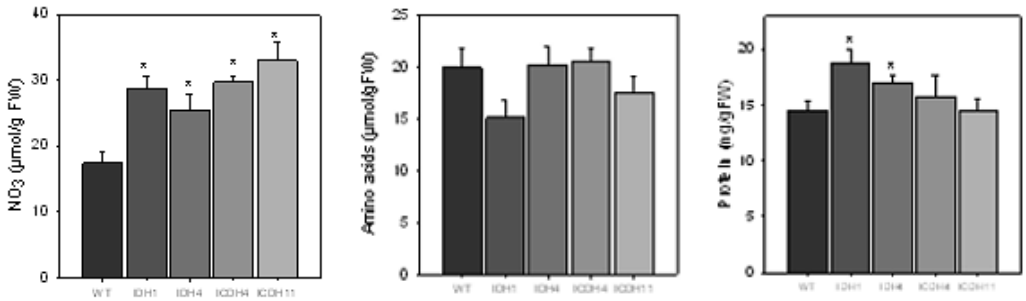


Figure 46: Nitrate, total amino acids and protein contents in leaves of NAD-IDH and NADP-ICDH transgenic tomato plants. The leaf material was harvested in the middle of the light period from five week old plants. . Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p< 0.05$) values obtained for each line in comparison to wild type as determined by the *t* test.

4.3.4.5 Photosynthetic pigment content

Given the above results, I next decided to evaluate the photosynthetic pigment content in the leaves, since these metabolites were reported to be important indicators of nitrogen deficiency (Gaude, et al., 2007). When the NAD-IDH and NADP-ICDH antisense leaves were quantified by the use of high-performance liquid chromatography (HPLC) they revealed a general decrease in pigment content (Figure 47). The level of both alpha- and beta-chlorophylls as well as violaxanthin was significantly reduced in all transgenic lines, while lutein and zeaxanthin were decreased in IDH4 line. Additionally, a significant reduction in lutein and neoxanthin was found in both NADP-ICDH lines. In contrary to these findings, beta-carotene and antheraxanthin remained unaltered in both sets of my transgenic plants.

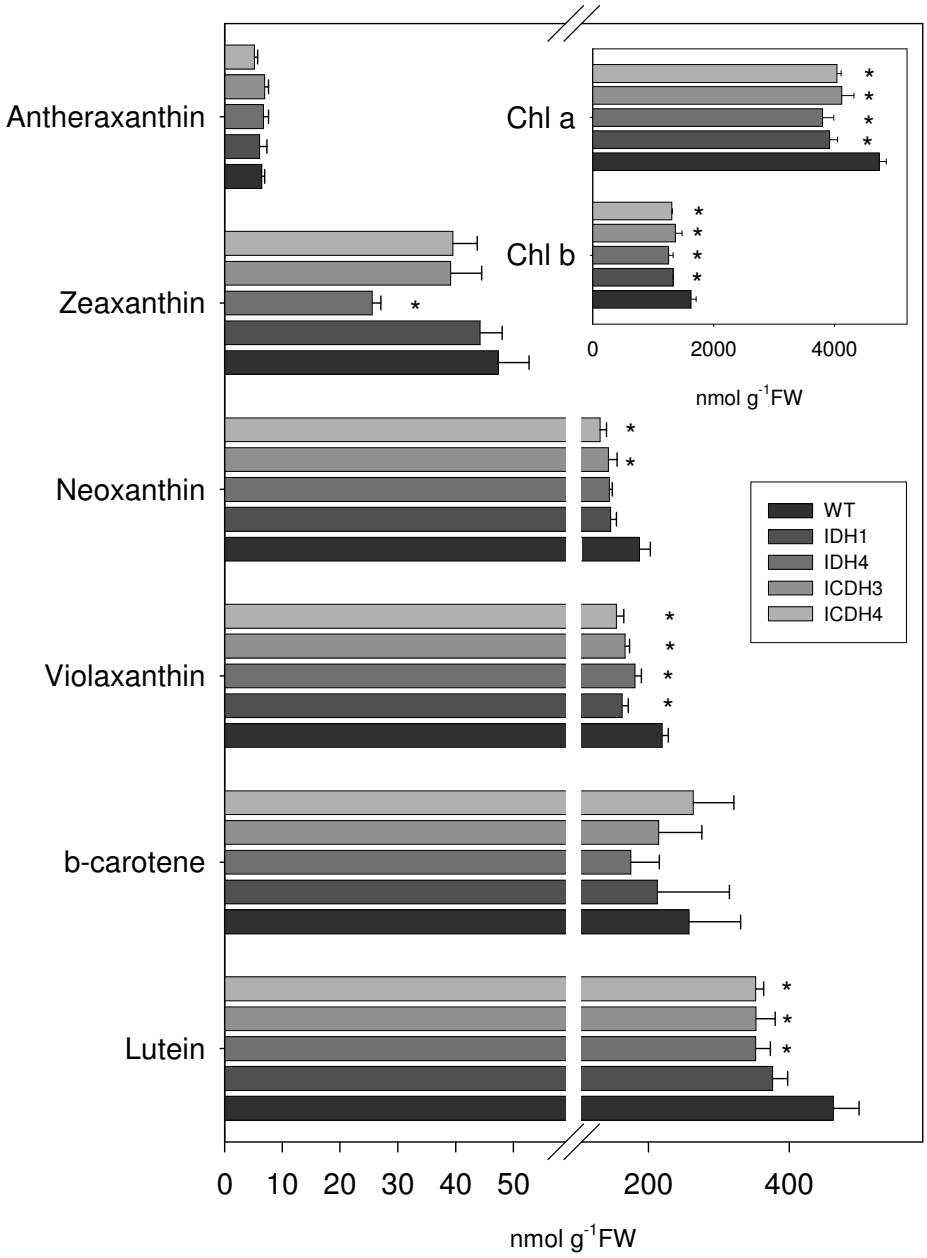


Figure 47: Pigment content in the leaves of NAD-IDH and NADP-ICDH transgenic tomato plants. Pigments were determined in six week old fully expanded source leaves harvested in the middle of the day. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p < 0.05$) values obtained for each line in comparison to wild type as determined by the *t* test.

4.3.5 Measurement of selected primary metabolism enzyme activities

Having analyzed the metabolite profile of NAD-IDH and NADP-ICDH plants I next investigated the maximal activities of a wide range of key enzymes of carbon and nitrogen metabolism in leaves by the use of robotized cycling assay (Table 8). Initially, I successfully confirmed the limited activity of the respective IDH/ICDH isoform in both sets of the transgenic genotypes.

Table 8: Enzyme activities determined in NAD-IDH and NADP-ICDH transgenic and wild type plants. The fully expanded source leaves were harvested six hours into the photoperiod from five week old tomato plants. Values are presented as mean ± SE of determination on six individual plants per line. Colour code indicates significantly ($p< 0.05$) increased (in red) or decreased (in green) values obtained for each line in comparison to wild type as determined by the *t* test. Abbreviation used were: PEP –phosphoenolpyruvate, MDH- malate dehydrogenase, GDH - glutamate dehydrogenase.

nmol min^{-1} g^{-1} FW	WT	IDH1	IDH4	ICDH3	ICDH4
Total IDH (NAD)	50,94 ± 2,90	26,28 ± 3,17	31,53 ± 4,41	42,94 ± 3,80	47,94 ± 5,25
Total ICDH (NADP)	975,69 ± 102,63	941,35 ± 54,24	1009,42 ± 60,75	664,06 ± 73,00	694,15 ± 96,76
Nitrate reductase	321,05 ± 32,76	356,52 ± 38,61	409,19 ± 45,32	260,557 ± 23,1752	269,925 ± 30,649
PEP carboxylase	1432,68 ± 96,30	1262,82 ± 43,10	1467,59 ± 46,06	1002,12 ± 89,94	1261,21 ± 96,76
Pyruvate kinase	1108,64 ± 94,20	1022,42 ± 77,10	1151,88 ± 101,68	774,31 ± 53,58	1114,15 4 83,26
Citrate synthase	138,41 ± 15,18	143,88 ± 11,95	145,43 ± 13,85	133,38 ± 16,17	148,92 ± 19,78
Aconitase	351,34 ± 40,94	326,15 ± 77,41	359,13 ± 46,05	334,80 ± 58,79	464,59 ± 71,03
MDH (NAD)	75304,24 ± 4558,54	77407,00 ± 5667,24	74292,62 ± 8425,27	81992,53 ± 5414,97	73068,12 ± 4914,24
Fumarase	5121,71 ± 853,18	2256,55 ± 545,91	2533,93 ± 592,69	3646,24 ± 583,53	3960,07 ± 350,61
GDH (NAD)	226,60 ± 25,16	332,24 ± 15,92	338,45 ± 40,54	289,35 ± 12,06	327,94 ± 13,82
Glycerate kinase	1963,57 ± 135,33	2087,79 ± 120,93	2530,41 ± 198,32	2346,30 ± 66,19	2226,96 ± 79,21
Total MDH (NADP)	5464,99 ± 184,15	5468,92 ± 173,55	5888,36 ± 260,32	5950,99 ± 134,791	5167,04 ± 245,29
Initial MDH (NADP)	795,72 ± 26,4	778,49 ± 45,75	939,14 ± 132,21	616,423 ± 19,5579	703,248 ± 17,736
MDH (NADP) activation	0,146 ± 0,003	0,144 ± 0,011	0,137 ± 0,002	0,10358 ± 0,002	0,136 ± 0,019

Moreover, the transgenic plants showed decrease in only one type of IDH/ICDH enzyme, confirming that the genetic modification was specific and that no compensatory effect was spotted on the enzymatic activity level. The enzymatic profile showed marked differences between the two sets of the transgenic plants. Interestingly, although my data suggested a reduction in capacity for nitrate assimilation in both transgenic genotypes, the nitrate reductase activity showed a decreasing tendency in NADP-ICDH lines, significant for ICDH3 line, but remained unaltered in the NAD-IDH transgenic leaves. Furthermore, the ICDH3 line was significantly decreased in the activities of both phosphoenolpyruvate carboxylase and pyruvate kinase, that were unaltered in NAD-IDH transgenics. Only two enzymes showed consistent direction of changes for both sets of transgenic plants. The activity of glutamate dehydrogenase (GDH) was significantly

increased in all genotypes and glycerate kinase was elevated in both NADP-ICDH lines and IDH4 line. Interestingly, although the previous data pointed into limited flux through the TCA cycle, the activities of enzymes involved in this pathway (CS, aconitase, NAD-MDH) remained at the wild type level, with the exception of fumarase, that showed two fold reduction in both NAD-IDH lines. Considering that the transgenic plants showed lower NAD(P)H levels, reduced pigment levels and efficiency of PSII, changes in the chloroplast redox state could be expected (Scheibe, 2004). Indeed, the quantified initial activity of NADP-dependent malate dehydrogenase (MDH) were significantly decreased in both NADP-ICDH lines, leading to limited activation state of the enzyme in ICDH3 line. These values remained unaltered in the NAD-IDH transgenic plants. The data indicate possible alterations in the chloroplast redox level in the transgenic leaves, emerging as a result of down regulation of the cytosolic, but not mitochondrial isocitrate dehydrogenase activity.

4.3.6 Transcript profiling of the NAD-IDH and NADP-ICDH transgenic leaves

I next carried out an investigation of the transcript profile of leaves of the transgenic lines. The analysis of mRNA content of several tomato isocitrate dehydrogenase genes was performed on both NAD-IDH and NADP-ICDH transgenic leaves by the use of the quantitative RT-PCR technique (Figure 48). The primers were designed in order to specifically target both mitochondrial NAD- dependent IDH genes (*Sl*IDH1 and *Sl*IDH2), as well as three NADP-dependent enzymes, located presumably in cytosol (*Sl*ICDH1 and *Sl*ICDH2) and in mitochondria (*Sl*ICDH3). As it would be expected, the results confirmed dramatic decrease in the transcript level of the two cloned tomato genes, namely *Sl*IDH1 gene in all transgenic NAD-IDH lines and *Sl*ICDH1 gene in all NADP-ICDH plants, respectively. Moreover, the expression of non-targeted isocitrate dehydrogenase isoforms remained unaltered in the NAD-IDH and NADP-ICDH transformants, which confirmed that the generated constructs were highly specific. Furthermore, none of the quantified genes demonstrated any compensatory effect on the transcript level that could possibly mask the effects of the genetic modification.

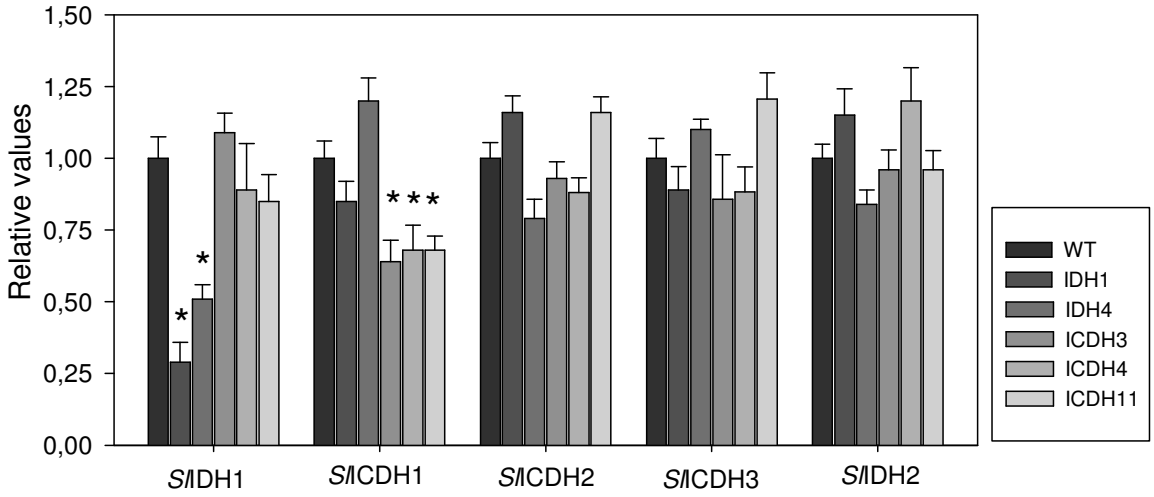


Figure 48: The relative transcript level of various isoforms of isocitrate dehydrogenase in tomato. The data was determined by RT-RT-PCR technique in fully expanded source leaves of five week old NAD-IDH, NADP-ICDH transgenic and wild type plants. Values are presented as mean ± SE of determination on six individual plants per line. An asterisk indicates significantly different ($p<0.05$) values obtained for each line in comparison to wild type as determined by the *t* test. Used abbreviations for *Solanum lycopersicum* genes: *Sl*IDH1 (TC193092), *Sl*ICDH1 (TC202045); *Sl*ICDH2 (TC164449); *Sl*ICDH3 mitochondrial ICDH like protein (TC196623); SlIDH2 (TC198615).

Broader transcript profiling of NAD-IDH plants (specifically line IDH4) was carried out by hybridization of leaf total cDNA with commercially available TOM1 microarray chips for Solanaceous species (Alba, et al., 2004). The data was statistically analyzed and visualized by the use of MapMan software (Urbanczyk-Wochniak, et al., 2006), which allows determination of over-representation of a particular functional category (BIN) by evaluating the responses of the all genes in each category compared with the overall response of all genes categories on the microarray (Usadel, et al., 2005). The normalized data revealed that the IDH4 transgenic line was not characterized by massive global changes in gene expression. According to the Wilcoxon Rank Sum test, with Benjamini Hochberg method applied, the most significant differences between transformed and wild type plants were found for a few categories (Figure 49). Firstly, the genes associated with the Calvin cycle were slightly down regulated in the IDH4 line, as exemplified by the six fold decrease in the expression of phosphoribulokinase. The second functional category that substantially changed contained carbonic anhydrases, where the expression of the genes corresponding to this enzyme were up to 2.5 fold reduced in the transgenic line. In the contrary to these findings, two gene categories showed substantial elevation of the transcript level. They grouped genes involved in catabolism of amino acids and metabolism of nucleotides. Moreover,

multiple genes performing regulatory functions were found to be significantly altered (data not shown). A general accumulation of transcript level was observed for genes associated with protein degradation, such as the Ser/Thr specific protein phosphatase, which increased six fold and 26S protease regulatory subunit VII, which increased 2.9-fold. Additionally, a substantial elevation in the transcript level was found for genes responsible for redox regulation, as exemplified by three fold increase of 2-oxoglutarate-dependent dioxygenase in IDH4 line.

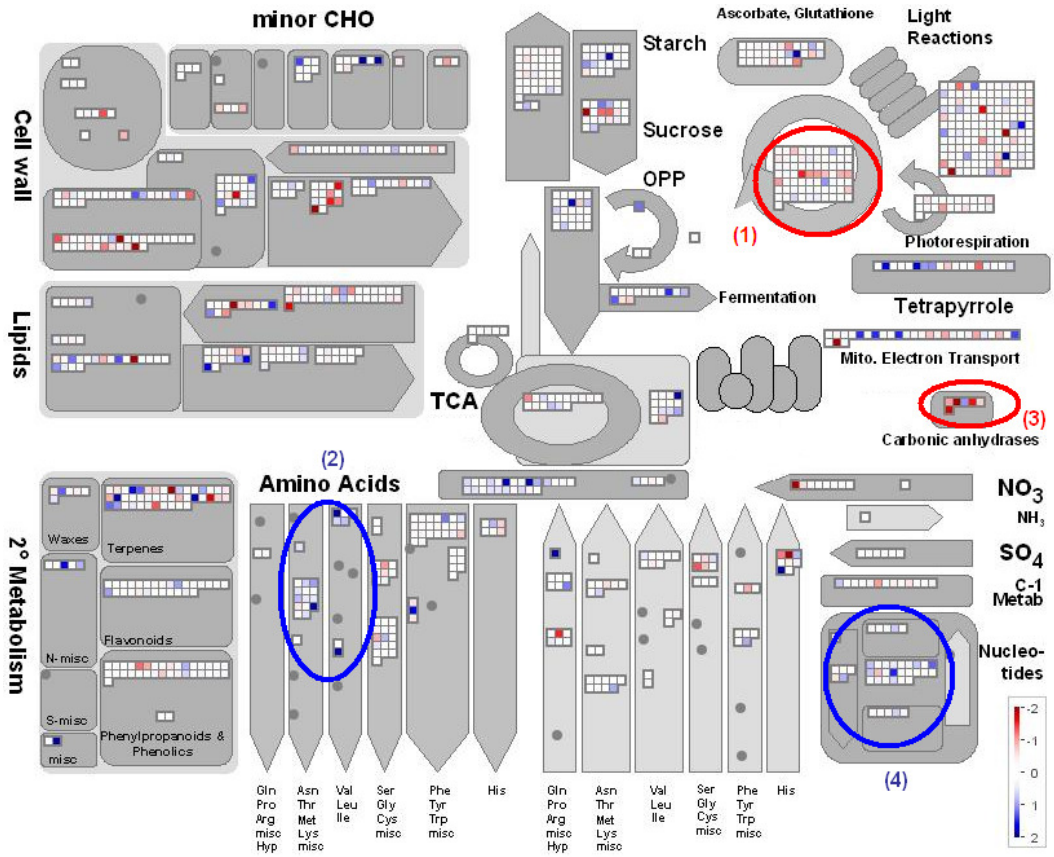


Figure 49: Transcript profiling in NAD-IDH leaves. The figure represents x fold increase (in blue) and decrease (in red) in the transcript levels of IDH4 line in comparison to wild type control for genes associated with metabolism. The applied colour scale is reproduced in the figure. The data represent mean value of four individual plants for each genotype. The numbers in the brackets indicate four functional categories (BINs), in which the genes showed significant differences to the response of all of the genes on the array. (1) BIN: 1.3.4: PS.calvin cyle; (2) BIN: 13.1.3.4: amino acid metabolism.synthesis.aspartate family.methionine; (3) BIN: 8.3: TCA / org. transformation.carbonic anhydrases; (4) BIN: 23.3, nucleotide metabolism.salvage.

4.3.7 Leaf metabolite response to nitrate starvation

The provision of carbon skeletons and reducing equivalents for nitrogen assimilation process is believed to be the major function of isocitrate dehydrogenase in plants. However, since the precise physiological role of each isoform of IDH re-

mains relatively poorly characterised, I decided to challenge both sets of transgenic tomatoes with conditions of nitrogen deficiency. Plants were grown hydroponically in nitrate limited medium, containing 0.4 mM NO_3^-, at the 900 $\mu mol^{-2}s^{-1}$ light intensity phytotrone for twenty days. These conditions induced chlorotic lesions, elevated root formation and dramatically decreased plant yield to a similar extent for all analyzed genotypes, including wild type control (data not shown). Although phenotypically undistinguishable the transgenic lines displayed several metabolic alterations. The majority of compounds, whose level were determinable by GC-MS were decreased in both transgenic genotypes in respect to wild type, however the decrease was definitely more pronounced in ICDH4 line (Figure 50). Interestingly, although the level of 2-OG decreased significantly in both transgenic lines in comparison to control plants, the dramatic decrease of this metabolite to the lowest level of detection was observed in ICDH4 line. This finding implies that the cytosolic NADP-ICDH isoform might be the major 2-OG supplier in plant metabolism and that the role of this enzyme becomes crucial for plant performance in stressful conditions, such as N deficiency. The consistent response to N limiting conditions in mitochondrial and cytosolic antisense plants was broken only for a few intermediates, namely sucrose, hexose phosphates and a handful of secondary metabolites. Similar discrepancies between both transgenics genotypes as compared to the wild type control were also observed for citrate and isocitrate. The sole metabolite that accumulated concomitantly in both transgenic sets was GABA. It is conceivable that this observation reflects this amino acid role in the typical plant response to the variety of environmental stresses (Bouche, et al., 2003). In summary, nitrate deficient conditions resulted in massive decrease in the level of the majority of primary C and N metabolites in both transgenic and control plants. Interestingly, the most dramatic alterations in leaf metabolite content, particularly in 2-OG level were observed in ICDH4 transgenic line. This finding suggests that NADP-ICDH transgenic plants were more susceptible to N starvation than NAD-IDH plants and that the cytosolic isoform may play an important role in provision of carbon skeletons for nitrate assimilation process in tomato.

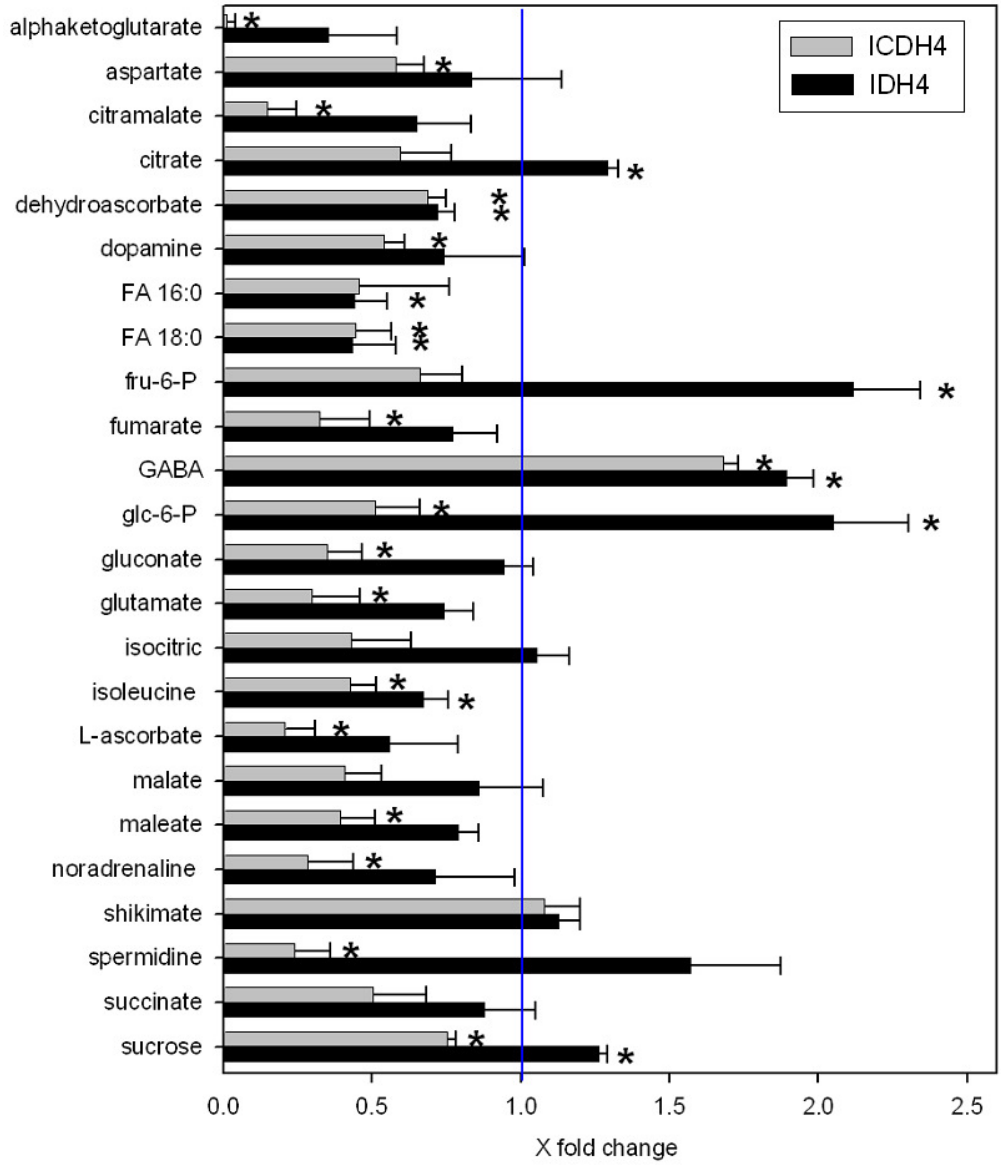


Figure 50: Metabolic response to nitrogen limiting conditions. The tomato transgenic lines IDH4, ICDH4 and wild type plants were subjected to nitrate deficient medium at the 900 $\mu mol^{-2}s^{-1}$ light intensity conditions for twenty days. The metabolite levels in harvested leaves were quantified by GC-MS technique and the data was subsequently normalised to the mean response calculated for wild type. The figure presents relative fold change of the values obtained for both transgenic genotypes after twenty days of nitrate deficiency. The straight line crossing X axis at one represents the level of metabolites quantified for wild type under the same stressful conditions. Values are presented as mean ± SE of determination on five individual plants per genotype.

4.4 Discussion and conclusion

The purpose of this work was to investigate the consequences of the inhibition of selected tomato isocitrate dehydrogenases on the metabolic and transcript content of the illuminated leaves, as well as on whole plant performance. Interestingly,

significant differences were found within two sets of the analyzed transgenic genotypes and these changes were mostly pronounced in leaf metabolic profile. Similarly to other transgenic plants with severely reduced cytosolic NADP-ICDH activity (Kruse, et al., 1998; Galvez, et al., 1999) and Arabidopsis mutants of mitochondrial NAD-IDH (Lin, et al., 2004; Lemaitre, et al., 2007), the photosynthetic performance of both sets of the transgenic tomatoes described here was not markedly changed. The rate of assimilation, transpiration, stomatal conductance and chloroplastic electron transport were invariant from wild type level. Interestingly, both transgenic genotypes revealed significant decrease in the maximal efficiency of photosystem II (Fv/Fm) and additionally, NAD-IDH plants were characterized by decrease in transcript level of several genes operating within Calvin cycle. Consistent with this alteration are the changes observed in pigment content of NAD-IDH and NADP-ICDH plants, such as the reduction in both alpha- and beta-chlorophylls, as well as violaxanthin and neoxanthin level. In contrast to other isocitrate dehydrogenase mutants (Zhao and McAlisterHenn, 1996; Lin, et al., 2004; Lemaitre, et al., 2007), the NAD-IDH transgenic tomatoes seem to possess altered mitochondrial respiration. First, the flux though TCA cycle was slightly decreased as indicated by elevated ratio of metabolized C1/C3:4-labeled glucoses in illuminated leaf discs. Secondly, on the metabolic level the NAD-IDH genotypes disclosed a significant decrease in organic acids content, presumably resulting from limited carbon flow through the TCA cycle and restricted production of their intermediates. Thirdly, the enzymatic activity of fumarase was two fold decreased in both NAD-IDH lines. The limitations in TCA cycle activity that occured in NAD-IDH plants is not surprising, since common regulation of the selected TCA cycle members has been already reported elsewhere (Kolbe, et al., 2006; Nunes-Nesi, et al., 2007a). Moreover, recent studies emphasized presumable associated inhibition of NAD-IDH and the sequential enzyme of the TCA cycle, namely 2-oxoglutarate dehydrogenase (OGDC) in potato tubers (Araujo, et al., 2008). In contrast to these findings, the NADP-ICDH plants remained unaltered in the level of citrate, isocitrate, 2-OG, succinate and fumarate. Moreover, the activities of none of the TCA cycle enzymes, including fumarase and NAD-IDH were changed in the NADP-ICDH transgenic leaves, hinting that the performance of this pathway was not influenced by the down regulation of cytosolic NADP-ICDH isoform.

Further alterations were observed in the photosynthetic metabolism of NAD-IDH and NADP-ICDH tomato plants. Although the NAD-IDH plants did not display any changes in the soluble sugar content of leaves, the NADP-ICDH lines revealed clear tendency towards decrease in sucrose and maltose level, in addition to significant reduction in glucose, fructose and hexose-6-phosphates level. Interestingly, the daily production of starch was significantly restricted in both sets of transgenic plants. Scheible and co-workers revealed that diurnal changes in starch and organic acids biosynthesis are synchronized to nitrate assimilation in plants (Scheible, et al., 1997b; Scheible, et al., 2000). More recent studies of C and N regulation have shown that nitrate acts as a potent signalling molecule, that is ca-

pable of redirecting carbon metabolism from sugar, fructan and starch biosynthesis into generation of organic acids (Stitt, 1999; Crawford and Forde, 2002; Forde, 2002b: and 2002a; Stitt, et al., 2002; Foyer, et al., 2003; Wang, et al., 2004). Indeed, down regulation of NAD-IDH and NADP-ICDH led to increased nitrate content in the transgenic leaves up to 168% and 189% of the wild type levels, respectively. Moreover, NAD-IDH lines were characterized by increased protein content but unchanged total amino acid pool sizes. On the other hand, the NADP-ICDH plants did not show any alterations in total amino acid and protein level, similarly to the transgenic potato plants displaying only 8% in the activity of the target protein, the cytosolic ICDH (Kruse, et al., 1998). The authors concluded that the potato cytosolic isoform of NADP-ICDH does not interfere with amino acid turnover, even when the plants were subjected to the conditions that induced leaf senescence. The results obtained here for the transgenic tomato plants suggested a considerable function for both mitochondrial and cytosolic isocitrate dehydrogenases in regulation of nitrate assimilation, however the precise physiological role of each enzyme is yet to be revealed.

Ammonia, which supplies the internal nitrogen cycle of leaves is generated by the mitochondrial decarboxylation of glycine to serine as part of the oxidative photosynthetic carbon cycle. Evidence has accumulated that the photorespiratory activity was elevated in the leaves of transgenic tomato plants described in this work, including the significant increase in glycerate kinase and limited accumulation of photorespiratory intermediates, such as glycolate, glycerate and glycine. Moreover, the directly measured carbon flux between glycine and serine was elevated in both NAD-IDH lines. When taken together, these data suggest that an upregulation of the photorespiratory pathway occurred in both transgenic genotypes. Since the increased flux through the glycine decarboxylase reaction would cause an elevated NH_4^+ level, this compound has to be immediately metabolised, as it is toxic for plants. Although the major route for assimilation of ammonium in plants cells is the GC-GOGAT cycle, plant cells also contain NAD(H)- dependent glutamate dehydrogenase (Coruzzi, 2003). The latter enzyme is mitochondrially localized and it catalyzes the reversible conversion of glutamate into 2-OG and ammonium (Dubois, et al., 2003). The increased photorespiratory performance in both sets of the transgenic tomato plants is interestingly mirrored by a significantly elevated activity of glutamate dehydrogenase (GDH).

2-OG is an important organic acid that plays a key role in metabolism by sustaining both the TCA cycle activity and nitrate assimilation processes (Scheible, et al., 2000; Hodges, 2002). Imbalances in 2-OG production and redox state in cytosol and mitochondria can influence broadly primary and secondary metabolism on the whole cell level and particularly nitrogen assimilation pathway. In plant cells there are multiple metabolic routes that lead towards the production of 2-OG for further respiratory and biosynthetic purposes. These include sugar respiration and amino acid transamination reactions catalyzed by several isoforms of isocitrate dehydro-

genases and aminotransferases (Scheible, et al., 2000; Hodges, 2002). Moreover, recent work performed on potato tubers (Aubert, et al., 2001) and Arabidopsis *gdh* mutants (Miyashita and Good, 2008) confirmed catabolic role of NAD-GDH enzyme in supporting TCA cycle with 2-OG, especially in carbon depleting conditions. Interestingly, multiple studies have shown that the internal 2-OG levels in plant cells were kept stable, regardlessly of analyzed conditions and genotypes (Scheible, et al., 2000; Matt, et al., 2001b: and 2001a; Matt, et al., 2002; Novitskaya, et al., 2002; Stitt, et al., 2002; Stitt and Fernie, 2003). Despite the importance of the 2-OG in plant biochemistry it is still not known where the major site of its production resides. It was suggested that the mitochondrial NAD-IDH enzyme, while serving as a regulatory step of the TCA cycle, can influence the stability of the 2-OG level and thus regulate the nitrogen assimilation process in plants (Lancien, et al., 1999; Stitt and Fernie, 2003; Abiko, et al., 2005a).

Results obtained for NAD-IDH transgenic tomato plants suggest that the mitochondrial isoform plays an important role in 2-OG production. The specific reduction of the mitochondrial NAD-IDH activity did not result in reduction of steady state 2-OG levels, although it significantly impaired the ^{13}C-label redistribution towards 2-OG in both transgenic lines. It can thus be hypothesized that the plants down regulated in the activity of mitochondrial NAD-IDH suffered from limitations in the carbon flux through the TCA cycle, which altered the production of 2-OG in the mitochondria (Figure 51). Consequently, a clear reduction in capacity for nitrate assimilation occurred in the transgenic leaves. The imbalance in cell carbon state led to several changes on the metabolic level, such as slight decreases in the level of amino acids, TCA cycle intermediates, photosynthetic pigments and starch, in addition to significantly increased level of nitrate and total protein. Furthermore, as a part of the compensatory reprogramming of metabolism, the transgenic plants displayed an up regulation of photorespiratory pathway. The increased flux through photorespiration may help to maintain the mitochondrial NADH homeostasis and stabilise glutamate levels. It is worth noting, that the NAD-IDH plants were characterized by the significant decrease in the NADH and NADPH levels. These pyridine nucleotides are direct products of various isoforms of isocitrate dehydrogenases and, when present in high concentration are capable of inhibiting these enzymes (McIntosh and Oliver, 1992; Hodges, et al., 2003). It is therefore likely that the activity of cytosolic NADP-ICDH would be de-inhibited in the transgenic plants. Indeed, the total NADP-ICDH activity in NAD-IDH plants remained unaltered. The cytosolic isoform would therefore contribute to 2-OG production, when supported by constant flow of citrate and/or isocitrate from mitochondria. Such a scenario is believed to take place in the illuminated leaves that suffer from high photorespiratory flux (Hanning, et al., 1999). It was shown that the reactions of the TCA cycle are severely inhibited by light and act at low level during the day (Budde and Randall, 1990; Tcherkez, et al., 2005). Moreover, it is believed that citrate is a major mitochondrial product in the light and that an alternative pathway exists that allows citrate to be exported from mitochondria in order to support 2-OG synthesis in the cytosol (Igamberdiev and

Gardestrom, 2003; Noctor, et al., 2006). Since the increased flux through the glycine decarboxylase reaction would result in elevated level of toxic ammonium in the mitochondrium, it is possible that these ions could be removed by the activity of mitochondrial GDH, that increased significantly in the transgenic tomato plants. Alternatively, it is plausible that the GDH enzyme was transferred to the cytosol in order to metabolize 2-OG provided by the cytosolic NADP-ICDH and ammonia released during both photorespiration and protein degradation. The function of this enzyme would include generation of glutamate and maintaining nitrogen metabolism. It has already been shown that the tobacco GDH enzyme can be induced in the cytosol in unfavourable conditions, such as elevated photorespiration and/or alterations in N source supply (Terce-Laforgue, et al., 2004). Another evidence of such a compensatory mechanism has been provided by antisense inhibition of ferredoxin-dependent glutamine-2-oxoglutarate aminotransferase (Fd-GOGAT) in tobacco (Ferrario-Mery, et al., 2002a). Following the transistion to the photorespiratory conditions, a temporary accumulation of 2-OG, glutamine and ammonia was observed in the transgenic leaves. Interestingly, diurnal changes in the level of these metabolites correlated well with the activity of NAD(H)-GDH, functioning in either aminating or deaminating direction, depending on the current metabolic requirements. This enzyme, accompanied by asparagine synthetase (AS) provided alternative routes for photorespiratory ammonia utilization in the tobacco plants suffering from inhibition of Fd-GOGAT. When taken all the evidence together, a clear mechanism for regulation of mitochondrial and cytosolic levels of pyridine nucleotides, isocitrate and 2-OG levels emerges (Igamberdiev and Gardestrom, 2003).

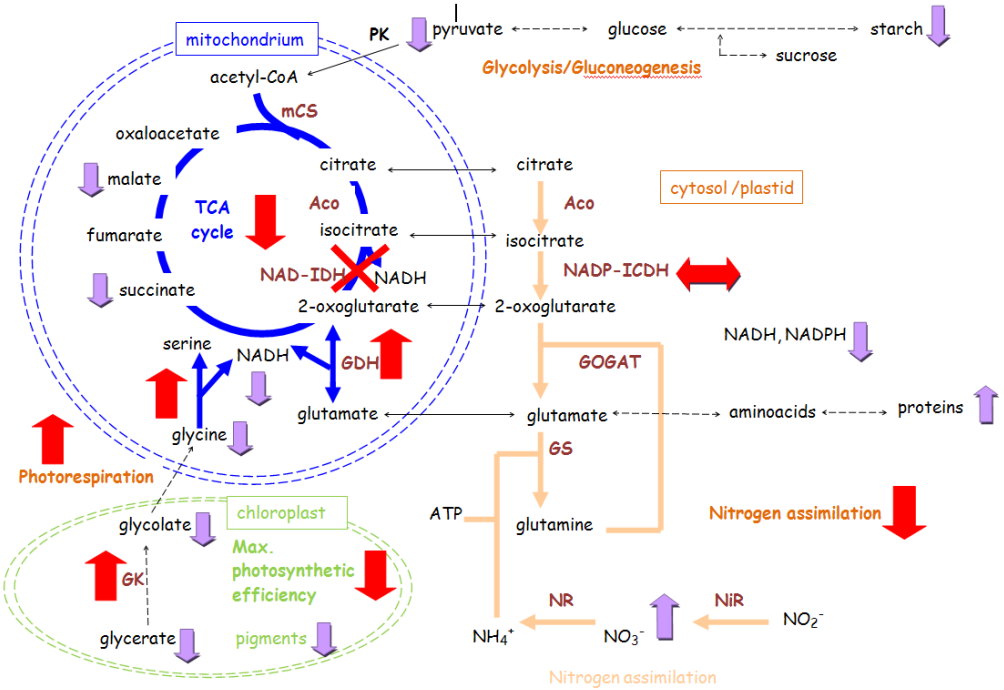


Figure 51: Hypothetic physiological and metabolic alterations occurring in the leaves of tomato plants down regulated in the activity of mitochondrially localised NAD-IDH (see full description in text). Decreased activity of NAD-IDH resulted in diminished flux through the TCA cycle and accumulation of its intermediates. Alterations in 2-OG production caused limitations in the flow through the nitrogen assimilation pathway, revealed by significantly increased nitrate and total protein level, as well as decreased starch accumulation in the transgenic leaves. The imbalance in the redox state of the cell, demonstrated as decreased level of NADH and NADPH would alter the activity of several enzymes, resulting in increased export of citrate outside the mitochondria in order to support nitrogen assimilation presumably via concerted action of cytosolic Aco and NADP-ICDH enzymes. The compensatory mechanism, aiming at stabilising pyridine nucleotides and 2-OG and glutamate levels would include elevated activity of photorespiratory pathway and increased activity of glutamate dehydrogenase (GDH). Additionally, the latter enzyme plays a role in removal of toxic ammonia accumulated by elevated glycine decarboxylation. Although the limitations in NAD-IDH did not inhibit photosynthetic carbon assimilation, the maximal efficiency of photosystem II (Fv/Fm) was significantly decreased in the transgenic leaves. These changes were accompanied by the consistent decrease in chlorophylls and xanthophylls content. Red arrows provide information of the flux through pathways, such as TCA cycle, photorespiration and nitrogen/ammonium assimilation and information of the enzymatic activities of following enzymes: NAD-IDH, NADP-ICDH, GDH and GK, that are either increased (arrows pointed up), decreased (arrows pointed down) or unaltered (vertical arrows). Arrows colored in violet represent accumulation (arrows pointed up) or decrease (arrows pointed down) of metabolites quantified in the leaves of NAD-IDH transgenic plants. Dashed black lines represent conversion between two metabolites that includes more than one reaction. Solid black lines represent transport of metabolites through membranes. The width of line is irrelevant. Abbreviation used were: GDH - glutamate dehydrogenase, GK - glycerate kinase, GS - glutamine synthetase, GOGAT- glutamate synthase, NR - nitrate reductase, NiR - nitrite reductase, PK –pyruvate kinase.

Similar mechanisms seem to compensate for limited activity of cytosolic isocitrate dehydrogenase isoform (Figure 52). Since multiple studies have stressed an importance of NADP-ICDH in supplying 2-OG for N assimilation pathway (Chen and Gadal, 1990; Fieuw, et al., 1995; Scheible, et al., 1997b; Galvez, et al., 1999; Stitt, 1999; Igamberdiev and Kleczkowski, 2003; Lemaitre and Hodges, 2006), it could perhaps be anticipated that the inhibition of NADP-ICDH is likely to influence N metabolism in the illuminated leaves. Indeed, the NADP-ICDH plants bore some characteristic symptoms of limitations in nitrogen assimilation. First, the starch production was severely impaired and the nitrate level was significantly elevated in all transgenic lines. Secondly, the NADP-ICDH leaves accumulated amino acids engaged in nitrogen metabolism, such as glutamine, asparagine, glutamate and GABA. This metabolic analysis showed that the decrease in NADP-IDH activity became a bottleneck of the pathway providing C for N assimilation, which consequently impacted on metabolite concentration. Thirdly, limitation in nitrate reductase activity, an enzyme directly involved in primary nitrate assimilation, was observed in both NADP-ICDH analyzed lines, significantly for ICDH3 line. Additionally, the NADP-ICDH plants were characterized by increased dry weight of roots when grown under optimized greenhouse conditions. Multiple studies have already provided convincing evidence that plants depleted in nitrogen source were characterized by altered architecture, including preferential root growth (Scheible, et al., 1997c; Stitt and Krapp, 1999) and increased lateral root formation (Zhang, et al., 1999; Zhang and Forde, 2000; Signora, et al., 2001; Forde, 2002a). Moreover, recent research has pointed in nitrate, as a signal that regulates shoot-root allocation and stimulates proliferation of lateral roots (Remans, et al., 2006; Walch-Liu, et al., 2006; Zhang, et al., 2007). Apart from limitations in N assimilation pathway, the decreased production of 2-OG by NADP-ICDH seemed to have no negative effect on the TCA cycle performance in my transgenic tomato plants. Nevertheless, massive changes were noticed in the pyridine nucleotides content, leading to significant drop down in the level of all NADH, NADP and NADPH and increase in NAD content. Such alterations in cell redox state could have dramatic effect on the activity of multiple cellular enzymes. It is therefore possible that the high NAD/NADH ratio quantified in NADP-ICDH leaves prevents inhibition of NAD-dependent TCA cycle enzymes, such as NAD-IDH or NAD-MDH or even activated NAD-GDH. The mitochondrial NAD-GDH enzyme is likely to serve a compensatory role, by supporting 2-OG production in the transgenic tomato plants displaying reduced activity of IDH/ICDH. Similarly, as mentioned above an up regulation of photorespiratory pathway was presumably another way of dealing with imbalances in the redox state of the cell that occurred in NADP-ICDH lines. Interestingly, application of all these compensatory strategies did not fully prevent alterations in the redox state of cytosol and organelles. Although both the maximal photosynthetic efficiency and pigment accumulation were significantly limited following inhibition of either cytosolic or mitochondrial IDH/ICDH in tomato, only NADP-ICDH plants revealed limitations in the activation state of NADP-MDH. It is a chloroplastic marker enzyme, which is controlled by the redox state and active only in

the light (Scheibe, 2004). The functional role proposed for this enzyme is the restoration of the NADP pool via a transport system known as the 'malate valve' (Scheibe, 1991: and 2004). By this process sufficient production of reducing equivalents for energy-consuming assimilatory processes is ensured in both plastids and the cytosol.

Interestingly, the NADP-ICDH transgenic plants were further characterized by alterations in the total carbohydrate level. Apart from the decrease in starch accumulation, these plants were characterized by mild reductions in the levels of maltose and sucrose, as well as significant decrease in hexoses and hexose-6-phosphates. These changes could be influenced by the limitations in the photosynthetic activity, in addition to alterations in the cellular energy balance. It is noteworthy that the data stay in great agreement with a decreasing trend in the activity of phosphoenolopyruvate (PEP) carboxylase and pyruvate kinase (PK), observed in both transgenic lines and significant for ICDH3 line. When taken together, these results are not particularly surprising, since it is known that sugar turnover is dependent on the rate of photosynthesis, glycolysis, gluconeogenesis and oxidative pentose-phosphate pathway (OPPP), which processes generate reducing equivalents and are redox-regulated. Recent studies of Arabidopsis nitrate transporters showed that the nitrate uptake and assimilation is regulated by glycolytic and OPPP-dependent carbon metabolism, although the signalling mechanism is still not known (Lejay, et al., 2008). Furthermore, common changes in the activity and/or transcript level of NADP-ICDH and other enzymes involved in carbon metabolism are not without precedence. Previous studies have shown that genes encoding enzymes responsible for production of carbon skeletons (such as PEP carboxylase, PK, NADP-ICDH, CS), sugar accumulation (AGPase, SPS) and assimilation of nitrogen (NR,NiR, GC, GOGAT) are reciprocally regulated (Scheible, et al., 1997a; Scheible, et al., 2000). Moreover, the transcription of these genes was dependent on availability of N source and internal nitrate level (Stitt, 1999). Thus, the results obtained for NADP-ICDH plants stay in agreement with current view of NADP-ICDH function in plants metabolism (Chen and Gadal, 1990; Fieuw, et al., 1995; Scheible, et al., 1997a; Lemaitre and Hodges, 2006), as well as co-regulation between C and N metabolism (Scheible, et al., 2000; Stitt, et al., 2002; Gray, et al., 2004; Leterrier, et al., 2007). The metabolic perturbations occurring in both sets of transgenic plants described here are presented in Figure 51 and Figure 52, respectively.

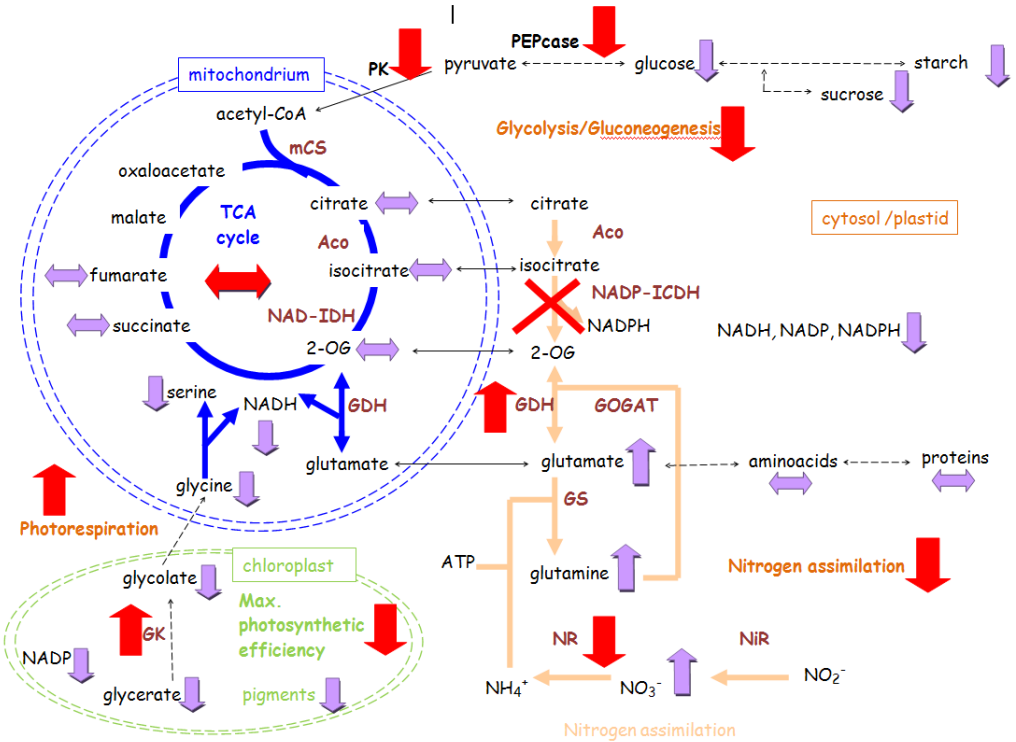


Figure 52: Hypothetic physiological and metabolic alterations occurring in the leaves of tomato plants down regulated in the activity of cytosolic NADP-ICDH (see full description in text). Decreased activity of NADP-ICDH resulted in limitations in the N assimilation pathway, manifested by diminished NR activity and accumulation of nitrate and selected amino acids and decrease in starch. These changes resulted in increased root formation in all NADP-ICDH lines growing in standard greenhouse conditions. The flux through the TCA cycle remained unaltered and did not influence steady-state organic acid content. Limitations in NADP-ICDH activity resulted in massive changes in cell redox state, visualized as decrease in total NADH, NADP, NADPH and increase in NAD level. This energy imbalance led to up regulation of compensatory routes, such as photorespiration, that could help to partially restore pyridine nucleotides level, as well as increased NAD-GDH activity, which can contribute to 2-OG production. Nevertheless, the compensatory strategies did not prevented imbalances in chloroplast redox state, that was quantified by significantly limited NADP-MDH activation state. Further changes in plastids included limited maximal photosynthetic efficiency (Fv/Fm) and decrease in the content of chlorophylls and xanthophylls. Interestingly, NADP-ICDH plants were also characterized by limitations in soluble carbohydrate content. The activity of two enzymes of the glycolytic pathway, namely PK and PEP carboxylase were significantly reduced in ICDH3 line. The genes encoding these enzymes are known to be co-regulated with the transcript level of NADP-ICDH and genes involved in N metabolism (such as NR), thus constituting a fine mechanism coordinating carbon and nitrogen provision in the plant cells. The data obtained here for NADP-ICDH transgenic plants confirm important role for cytosolic isoform in provision of 2-OG for nitrate assimilation process in tomato leaves. Red arrows provide information of the flux through pathways (TCA cycle, photorespiration, glycolysis and nitrogen/ammonium assimilation) and enzymatic activities (NADP-ICDH, GDH, GK, PK, PEP carboxylase and NR), that were either increased (arrows pointed up), decreased (arrows pointed down) or unaltered (vertical arrows). Arrows colored in violet represent accumulation (arrows pointed up) or decrease (arrows pointed down) of metabolites quantified in the leaves of NADP-ICDH transgenic plants. Type of lines were used as in Figure 51. Abbreviation used were as above; PEPcase- phosphoenolpyruvate carboxylase.

The results described in this work showed that the influence of isocitrate dehydrogenase enzymes is not only limited to the mitochondrial or cytosolic metabolism but it spans through all organelles and extends to other cellular processes, such as nitrogen assimilation, photosynthesis and photorespiration. The regulation of these pathways is often achieved by alterations in the redox homeostasis. The NADH and NADPH levels are known to play a vital role in mitochondrial respiratory metabolism (Noctor, et al., 2007) and under many different conditions free mitochondrial NADH levels are kept constant (Kasimova, et al., 2006). Research of Dutilleul and co-workers (2003a; 2003b; 2005) used the tobacco CMS I mutant to reveal that NADH availability is a critical factor influencing the rate of nitrate assimilation and that NAD status plays a crucial role in coordinating ammonia assimilation with the anaplerotic production of carbon skeletons. This work provided an important evidence of mitochondrial NAD-IDH being directly involved in 2-OG production for ammonia assimilation purposes (Dutilleul, et al., 2005). Interestingly, a very different metabolic profile was found in nitrate reductase (NR)-deficient plants. In this mutant decreased citrate to 2-OG ratio promoted by high leaf nitrate was associated with a specific induction of cytosolic NADP-ICDH (Scheible, et al., 1997a). The authors proposed that nitrate can act as a signalling molecule, by repressing starch accumulation and activating organic acid production via cytosolic NADP-ICDH in order to meet the carbon requirement of N assimilation pathway. Furthermore, the tobacco wild type plants had excess capacity for nitrate assimilation and utilised this potential only for a short time each day. This study provides an explanation to the fact that the transgenic tomatoes analyzed here were able to grow and reach comparable to wild type height in the same time frame, even though they struggled with limited flux though TCA cycle and nitrogen assimilation pathways (NAD-IDH plants) or even with significantly decreased activity of nitrate reductase (NADP-ICDH plants).

Interesting results concerning the functional role of both NAD- and NADP- dependent isocitrate dehydrogenase enzymes in plant metabolism were obtained by studying the tomato IDH/ICDH lines following subjection to nitrogen deficiency. Both transgenic genotypes cultivated in low nitrogen liquid medium in high light conditions presented growth retardations typical for N deficiency and were phenotypically indistinguishable from control plants. Nevertheless, the drop in the metabolic content of NAD-IDH leaves, as compared to the wild type level was far less dramatic than in case of NADP-ICDH plants. This was particularly noteable in case of 2-OG, which fell to below the detection level in NADP-ICDH plants. The fact that these transgenic tomatoes responded more sharply to limitations in nitrogen supply hints towards increased susceptibility of NADP-ICDH plants to nitrate stress. As previously postulated (Chen and Gadal, 1990; Scheible, et al., 1997a), I therefore conclude that this isoform is more relevant for nitrogen assimilation process in plant leaves. Since, it was shown that under normal growth conditions, the NADP-ICDH plants did not reveal any alterations in the enzymatic or transcript level of NAD-IDH, it becomes apparent that these metabolic alterations were caused directly by the down regulation of the cytosolic isoform of isocitrate

dehydrogenase. Hence, this isoform bears a greater responsibility for supplying 2-OG for the nitrogen assimilation process than a mitochondrial one. Moreover, the function of NADP-ICDH must be crucial in N metabolism of tomato, as it cannot be easily compensated in the event of limited nitrate provision. Higher susceptibility of cytosolic NADP-ICDH to N limited conditions is in accordance with the recently proposed role of this isoform in defensive response against variety of environmental stresses (Leterrier, et al., 2007). By contrast the NAD-IDH activity seems to be important for sustaining mitochondrial respiration and TCA cycle activity, although metabolic perturbations occurring in optimized N conditions hints towards an additional, albeit minor role in supporting nitrate assimilation.

When the NAD-IDH tomatoes were compared with Arabidopsis plants containing only 8% of wild type mitochondrial NAD-IDH activity (Lemaitre, et al., 2007) very few similarities were found. Surprisingly, the Arabidopsis mutants displayed no major metabolic alterations and no specific phenotype when grown in optimized greenhouse conditions. However, low light intensity and liquid culture conditions induced a massive growth retardation and accumulation of organic acids and sugars in rosette leaves. The authors of this study proposed a model involving differences in reducing power and ATP supply by TCA cycle, depending on growth conditions. Interestingly, the metabolite content of NAD-IDH tomato plants subjected to N depletion in hydroponic culture resembled that of Arabidopsis NAD-IDH mutants grown in low C conditions of liquid culture. That said, both sets of plants accumulated citrate and hexose-6-phosphates in their leaves. This finding demonstrates that the mitochondrial NAD-IDH isoform became limiting for TCA cycle performance upon nutrient deficient conditions. When taking all data together, it could be concluded that the blockage in IDH step of TCA cycle caused different aberrations in tomato and Arabidopsis plants and that various plant species deal differently with such perturbation of metabolism. While the deficiency in the Arabidopsis enzyme had little effect on plant performance and influenced the TCA cycle efficiency only under conditions of stress, the tomato enzyme seems to possess a wider function in metabolism. It is required to maintain correct carbon flow through the TCA cycle in both N rich and N shortage conditions and additionally, its control is extended over the N assimilation pathway in tomato. Importantly, the total number of NAD-IDH tomato genes is still not known. Moreover, their regulation, including several post transcriptional modifications remains unclear and may vary between tissues and species. It is noteworthy that the transgenic plants described here differed from Arabidopsis mutants in level of repression of the target enzyme, but also in the range of metabolic changes, as exemplified by 1.5 fold accumulation of citrate in tomatoes in comparison to 26 fold increase in idhv and 5 fold increase in idhii mutants. Such discrepancies in the level of response between different Arabidopsis lines depend on the IDH gene affected. The most dramatic alterations in comparison to wild type were presented by idhv line repressed in catalytic subunit of NAD-IDH, whereas two other mutants with inhibited activity of the regulatory NAD-IDH subunit presented generally much more mild changes when compared to control

plants. Similarly, the NAD-IDH tomatoes were also disrupted in the performance of the regulatory subunit, which shared the highest sequence similarity to *IDH I* and *IDH II* Arabidopsis genes.

Several recent studies have demonstrated that other mitochondrially localised proteins have a profound impact on nitrogen metabolism (Dutilleul, et al., 2005; Pellny, et al., 2008). Additional evidence has been provided by addition of rotenone, a specific inhibitor of complex I, to Arabidopsis cell suspension cultures (Garmier, et al., 2008). This study revealed that 2-OG production is rapidly reduced after application of the inhibitor and it remains decreased for at least 16 hours. Moreover, important evidence for the role of mitochondrial metabolism in the maintenance of optimal photosynthetic performance have been provided for uncoupling protein 1 (Sweetlove, et al., 2006) and alternative oxidase (Strodtkotter, et al., 2009), which appear to play a role in photorespiration. Another example of a close interdependence between mitochondrial respiration and cellular redox balance was provided by Morgan and co-workers (Morgan, et al., 2008). Arabidopsis plants limited in the activity of mitochondrial Mn-superoxide dismutase were characterized by inhibition in flux through the TCA cycle, particularly at the aconitase and mitochondrial NAD-IDH steps.

Apart from the metabolic variations, the transgenic lines possessing limited activity of either mitochondrial or cytosolic isocitrate dehydrogenase showed several similarities at the physiological level and in plant architecture. That said, each set of plants displayed mild but corresponding phenotype, revealed in largely unaltered vegetative growth and strongly limited fruit production. Generally, all transgenic lines demonstrated a relatively unaltered biomass accumulation of all organs, with the exception of roots. Whilst the NAD-IDH plants showed minor decreasing tendency in root formation, the NADP-ICDH plants were characterized by significantly increased root dry weight. Considering the fact that elevated root biomass is a well known plant response to limitations in nitrogen availability, this finding is particularly interesting as it hints towards restrictions in nitrogen assimilation occurring in plants inhibited in NADP-ICDH activity. Oppositely to these discrepancies in root formation, both sets of transgenic plants were further characterized by concordant dramatic reduction in fruit production. The explanation which I favour for the decrease in fruit yield observed in the transgenic tomato plants is either an alteration in fruit sink strength, caused by altered number of fruits (sink size) or a reduced competitive ability of the fruits to import assimilates (sink activity) (Herbers and Sonnewald, 1998). Since the generated number of fruits per plant was increased by the down regulation of NADP-ICDH but it was not influenced by the inhibition of NAD-IDH, it is possible that the mechanism underlying the limited fruit production might be different for both sets of the transgenic tomatoes. Given that the constitutive CaMV 35S promoter, used to generate the chimeric constructs would presumably affect the activity of isocitrate dehydrogenase in all plant organs, it is impossible to dissect the impact of the ge-

netic perturbation on fruit metabolism *per se*, which would require a fruit-specific promoter. For this reason, I cannot distinguish to date whether the loss of fruit yield in my transgenic plants was due to sink or source effects. It was shown that in tomato both cases are possible (Obiadalla-Ali, et al., 2004; Nunes-Nesi, et al., 2005b; Zanor, et al., 2009). Interestingly, although the modifications in flower development and/or fruit production are common for TCA cycle mutants (Landschutze, et al., 1995a; Carrari, et al., 2003a; Yui, et al., 2003; Nunes-Nesi, et al., 2005b; Nunes-Nesi, et al., 2007a), neither of plants possessing decreased isocitrate dehydrogenase activity (Kruse, et al., 1998; Galvez, et al., 1999; Lemaitre, et al., 2007) was suppressed in fruit formation. The authors concluded that neither IDH/ICDH isoform are essential for plant survival. Nevertheless, according to the literature survey, proper functioning of cytosolic NADP-ICDH may have large impact on tomato fruit metabolism and development, which could perhaps influence the fruit number of NADP-ICDH tomato plants. It was shown that the cytosolic enzyme is predominantly active out of all NADP-dependent isoforms and highly abundant in flowers and fruits (Leterrier, et al., 2007). The cytosolic isoform was suggested to be the main one involved in 2-oxoglutarate production in the ripening tomato fruits (Palomo, et al., 1998). More recently, the changes in cytosolic NADP-ICDH transcript level were found in ripening fruits to be highly dependent on farming conditions, stressing the role of this enzyme in providing a linkage between carbon metabolism in fruits and nitrogen sensing in roots of tomato plants (Neelam, et al., 2008).

In summary, this work was dedicated to determination of the significance of two major isocitrate dehydrogenase isoforms in tomato leaf metabolism. Multiple studies were designed aiming at the elucidation of an actual physiological role of each isoform of isocitrate dehydrogenase, however this task has not been to date achieved. My data showed that minor changes in activity of mitochondrial NAD-IDH and cytosolic NADP-ICDH isoforms in tomato resulted in major metabolic consequences in the transgenic leaves, but had little effect on global patterns of transcription, growth or plant performance. That said, it could be concluded that high activity of neither of the tomato IDH/ICDH isoforms is essential for plant survival and the introduced limitations could be effectively overcome by up regulation of compensatory mechanisms. These findings are in accordance with current view of IDH/ICDH function in plant tissues and provide confirmation for the results obtained in other eukaryotic mutants (Lancien, et al., 1998; Lemaitre, et al., 2007; Leterrier, et al., 2007). Additionally, my data demonstrated an important role for both IDH/ICDH subunits in generation of carbon residue to fuel N assimilation pathway. Apart from consistent metabolic changes in starch and nitrate level that occurred in both sets of transgenics, the NADP-ICDH plants showed direct alterations in the level of intermediates and activity of enzymes associated with N metabolism. Moreover, these modifications were reflected by phenotypic changes, such as increased root formation in standard growth conditions. These results suggest that the NADP-ICDH transgenic plants were more severely impaired in the ability to assimilate nitrate. Additionally, the nitrate deficiency stress led to much

more dramatic decrease of major C and N metabolites in ICDH4 line than in IDH4 line, particularly in the level of 2-OG. Considering the fact that the NAD-IDH plants also revealed minor alterations in the N metabolism, even though the activity of NADP-ICDH was not suppressed in these plants, I conclude that in tomato both IDH/ICDH enzymes are standing at the cross road of carbon and nitrogen metabolism, however the cytosolic isoform seems to be the major donor of carbon skeletons for the nitrate assimilation pathway. When taken together, these results correspond to current understanding of coordinated regulation of C and N metabolism (Gray, et al., 2004; Lemaitre and Hodges, 2006; Leterrier, et al., 2007; Scheible, et al., 2000; Stitt, 1999). More experiments need to be performed to fully characterise each set of transgenic plants in order to widen our understanding of the physiological role of each subunit in plant biochemistry.

5 FINAL DISCUSSION AND SUMMARY

The TCA cycle is a respiratory metabolic pathway of central importance for all living organisms. It is composed of a serial of enzyme-catalyzed chemical reactions that are mainly located in mitochondria and remain reciprocally dependent (Carrari, et al., 2003b). Despite the crucial role of this pathway in plant metabolism, the precise physiological function of the TCA cycle has not yet been fully elucidated thus far (Siedow and Day, 2000; Nunes-Nesi, et al., 2007b; Sweetlove, et al., 2007; Nunes-Nesi, et al., 2008; Van der Merwe, et al., 2009b). In this project the activities of two major players in mitochondrial TCA cycle, in addition to one cytosolic enzyme, were independently inhibited and the intracellular and physiological responses to these perturbations were carefully investigated. Since the cellular ability to respire remains a foundation for sustaining life on Earth, any modifications in this process would be expected to have tragic consequences on the performance at both single cell and organismal levels. Then why did the genetic modification of the TCA cycle in my transgenic tomatoes not result in lethality or even in a dramatic phenotype?

The answer to this question includes the existence of unique features in plant mitochondria. During the process of evolution plants have gained increased flexibility of respiratory pathways (Mackenzie and McIntosh, 1999) that assured their survival in unfavorable environmental conditions. In addition to possessing multiple entry points to the respiratory pathway from sucrose and starch, plants are frequently able to accomplish the same step in a metabolic pathway in a variety of different ways. The metabolic bypasses for blockages in various steps of TCA cycle can be generated by utilizing glyoxylate cycle, shikimate cycle, GABA shunt and handful variety of other reactions, many of which may still remain undiscovered. Moreover, a compensatory role may also be intercepted by up regulation of another isoform of the inhibited enzyme located either in separate cell compartment or in the same organelle but playing a distinct physiological function. Furthermore, activation of other enzymes catalyzing similar biochemical reactions and carrying structural similarity to the inhibited enzyme can not be excluded.

The up regulation of the peroxisomal isoform of citrate synthase (pCS) was shown to be present in tomato leaves with inhibited activity of mitochondrial CS. Following increase in the transcript level of pCS gene that was validated in the transgenic leaves, the possibility of higher performance of the whole glyoxylate cycle exists. The elevated activity of this pathway was proposed on the basis of significantly increased flux from citrate and isocitrate to succinate that in both transgenic lines was two fold higher than in control plants. It was demonstrated that succinate, generated in the peroxisome can be further transported through the succinate-fumarate translocator (Catoni, et al., 2003) into mitochondrion and utilized within TCA cycle. The enzyme catalyzing such reaction - isocitrate lyase is consi-

dered to serve an anaplerotic function in plants (Eastmond and Graham, 2001). Furthermore, recent work on Arabidopsis mutants have proven in seeds that peroxisomal CS is required for fatty acid respiration via the glyoxylate cycle (Pracharoenwattana, et al., 2005). Interestingly, this CS isoform was significantly up regulated at the transcript level in my CS transgenic tomato plants. The authors showed that the major metabolite exported from peroxisomes in plants is citrate, which can subsequently be imported into mitochondria by carboxylic acid transporter (Picault, et al., 2002) in order to fuel the TCA cycle. However, whether such scenario is likely to occur in fully developed leaves remains unknown.

Since the glyoxylate cycle enables to omit the IDH step in the TCA cycle, it is also possible that this bypass reaction could be activated in NAD-IDH and /or NADP-ICDH transgenic plants, although direct evidences are yet to be gathered in support of this theory. However, high activity of glyoxylate cycle during gluconeogenesis was shown to be correlated with inhibition in mitochondrial NAD-IDH and following restrictions in carbon flux through the TCA cycle (Hill, et al., 1992; Falk, et al., 1998). Nevertheless, the up regulation of glyoxylate cycle is not very likely to occur in NAD-IDH plants, as the metabolic data showed unaltered level of sucrose and fatty acids in the transgenic leaves, in addition to lack of major changes in carbon flux between glyoxylate cycle intermediates (data not shown). In contrast to these findings, the NADP-ICDH displayed elevated sucrose content and significant decrease in palmitic and stearic acids, which may hint towards limitations in fatty acid respiration. Perhaps, it could be correlated with the activity of cytosolic aconitase, that was shown to metabolize citrate of peroxisomal origin within the glyxolate cycle in etiolated pumpkin cotyledons (Hayashi, et al., 1995). This theory is however purely speculative, since high activity of glyoxylate cycle is usually associated with seeds and early stages of plant development and the functional role of this pathway in plants is still a topic of a debate (Eastmond and Graham, 2001; Smith, 2002; Pracharoenwattana, et al., 2007; Pracharoenwattana and Smith, 2008; Smith, et al., 2008).

Additional possible bypass of CS deficiency may occur via acetyl CoA hydrolase, which catalyzes hydrolysis of CoA moieties within plant mitochondria (Zeiher and Randall, 1990). This enzyme was shown to be involved in hydrolyzing surplus of acetyl CoA that accumulates during oxidative metabolism in animal tissues (Soling and Rescher, 1985), therefore it is tempting to propose similar function of this enzyme in plants. The hypothetic upregulation of acetyl CoA hydrolase in CS-antisensed plants would probably influence the activity of acetyl CoA synthetase in leaves, since the latter enzyme was shown to respond to alterations in acetyl CoA level and was significantly increased in transgenic tobacco plants expressing yeast acetyl CoA hydrolase in mitochondria (Bender-Machado, et al., 2004).

The alternative route for the TCA cycle reactions in plants is also afforded via the GABA shunt (Satyanarayan and Nair, 1986; Bouche, et al., 2003). It is responsible for production of gamma-aminobutyric acid (GABA) and its further conversion to succinate that re-enters the TCA cycle. The pathway is composed of three enzymes: the cytosolic glutamate decarboxylase (GAD) and the mitochondrial enzymes GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), which activity was shown to be enhanced in response to biotic and abiotic stresses (Snedden and Fromm, 1999; Bouche, et al., 2003; Shelp, et al., 2003). In plants, GABA is produced almost exclusively by GAD and its activity is modulated by Ca2+/calmodulin (Snedden, et al., 1995). Initially, the hyper activation of GABA shunt in any set of my transgenic plants was not expected, as the pathway provides the bypass of subsequent TCA steps and was shown to efficiently compensate inhibited succinyl-CoA ligase in tomato (Studart-Guimaraes, et al., 2007). Nevertheless, both IDH/ICDH transgenic plants were characterized by significantly increased activity of the first enzyme of this cycle, namely glutamate dehydrogenase (GDH). Additionally, NADP-ICDH plants were found to possess increased level of GABA shunt intermediates, such as GABA, Glu and Gln.It is therefore possible, that at least some of the steps of GABA shunt were activated in order to support generation of 2-OG or, alternatively to detoxify high levels of ammonium produced by increased photorespiration in both sets of these transgenic plants.

The transgenic tomato plants down regulated in the activity of TCA cycle enzymes, namely CS and NAD-IDH revealed limitations in the carbon flux through this pathway. In such conditions the supportive up regulation of mitochondrial NADP-ICDH may occur, as it was proposed by Gray and co-workers (2004). This isoform was shown to supply the performance of NAD-IDH in conditions that create a metabolic imbalance between respiratory carbon metabolism and electron transport and therefore regulate flux through the TCA cycle. Consequently, increased activity of mitochondrial NADP-ICDH would cause further changes in the reducing equivalent level and composition, which would likely influence the oxidative phosphorylation system of the cell. Indeed, both mitochondrial type II NAD(P)H dehydrogenases and alternative oxidase (AOX) were proposed to act as reductant overflow enzymes by reoxidizing surplus NAD(P)H to oxygen (Millenaar and Lambers, 2003). More recently, the external NADP-dehydrogenase was shown to regulate the pyridine nucleotide content in tobacco, although the overexpression of this enzyme did not significantly influenced growth rate, chloroplast MDH activation state or xanthophylls content in the transgenic plants (Liu, et al., 2008). Interestingly, the Arabidopsis alternative respiratory pathway were found to be regulated by nitrate and ammonium signaling (Escobar, et al., 2006). These pathways of electron transport do not contribute to respiratory ATP production and are independent of cellular adenylate status, thus they prevent inhibition of the TCA cycle under high ATP/ADP conditions (Noctor and Foyer, 1998), however the adenylate content in all my transgenic plants resembled wild type level. The reducing power overflow that was overcome by induction of energy

bypass proteins appears often in photorespiratory conditions. The common changes in the activity of AOX and isocitrate dehydrogenase are not without precedence. High levels of the activated, reduced form of AOX were already observed in the leaves of tobacco that over-expressed mitochondrially located NADP-ICDH (Gray, et al., 2004). Moreover, AOX expression in tobacco was also induced by exogenous application of selected TCA cycle intermediates, which proves that the enzyme responds to changes in level of citrate, malate and 2-oxoglutarate. Gray and co-workers (2004) suggested a supportive role for mitochondrial NADP-ICDH, to maintain the flux through TCA cycle and furthermore activate AOX in conditions of metabolic imbalance. This scenario could take place in any of my transgenic tomato genotypes and could therefore provide an explanation for elevated mitochondrial respiration rates quantified in CS antisense plants. The RT-PCR results of IDH/ICDH plants did not show a significant elevation of the transcript level of *Sl*ICDH3 (TC196623), which showed highest sequence similarity to mitochondrial NADP-ICDH proteins from other plant species. However, the tomato genome is still not fully elucidated, thus the existence of other genes encoding the mitochondrial NADP-ICDH may occur. The compensatory up regulation of another IDH/ICDH isoform in my transgenic plants cannot be therefore excluded.

In this study I present the functional analysis of tomato plants exhibiting limited activity of mitochondrial citrate synthase and two isoforms of isocitrate dehydrogenase, mitochondrially and cytosolically located ones. Interestingly, the transgenic tomato lines characterized by decreased activity of both mitochondrially located enzymes shared a similar response towards introduced system imbalance. That said, CS and NAD-IDH antisense lines both revealed alterations in ^{14}C-glucose and ^{13}C-pyruvate radiolabel distribution, pointing in a restricted carbon flow through the TCA cycle and elevated flux through photorespiration. Apart from the changes in respiratory performance, the transgenic plants were slightly impaired in nitrate assimilation. Surprisingly, the metabolic profile of these plants was rather distinct, with only few exceptions. They included a common decrease in the level of organic acids involved in the TCA cycle and photorespiration, as well as accumulation of nitrate and reduction in chlorophylls and xanthophylls. Nevertheless, both sets of the transgenics displayed some similarities in the transcript profile. The significant decrease in mRNA level of genes encoding carbonic anhydrases were accompanied by elevation of transcript level of genes involved in starch and aspartate-derived amino acids metabolism. All these changes can be presumably associated with the decrease in TCA cycle performance and following alterations in both 2-OG level and redox balance within mitochondria. It is noteworthy, that both targeted enzymes do not simply participate in TCA cycle, but they are believed to represent rate-limiting steps of the TCA cycle and flux-limitation points in plant respiration (Chen and Gadal, 1990; Hill, et al., 1992; Falk, et al., 1998; Popova and de Carvalho, 1998). The data I obtained suggest that the activity of enzymes that possess similar function and act within one pathway are reciprocally dependent and co-regulated. Furthermore, the regulation of

functionality of whole pathway level remains superior over the regulation of activity of each single member of this pathway, which is in accordance with current view concerning regulation of plant metabolism in vivo (Dennis and Blakeley, 2000).

Surprisingly, the modifications occurring in my transgenic CS and NAD-IDH plants are distinct from those previously described in plants limited in the activity of other TCA cycle members. Firstly, the transgenic and mutant tomatoes displaying decrease in selected TCA cycle enzymes (Carrari, et al., 2003a; Nunes-Nesi, et al., 2005b; Nunes-Nesi, et al., 2007a; Studart-Guimaraes, et al., 2007) were characterized by either improvement or impairment of photosynthetic carbon assimilation, which was unaltered in my plants. Secondly, none of these plants revealed any inhibition in nitrogen metabolism, whereas both CS and NAD-IDH plants displayed symptoms of decreased N assimilation ability. Nevertheless, all above mentioned plants inhibited in TCA cycle activity, including my transgenics revealed both consistently lowered flux through the cycle and mild phenotypic perturbations in fruit production. An important feature that distinguishes my lines from the above mentioned transgenic plants is the fact that my plants were impaired in the activity of enzymes acting within the 'initial', decarboxylating part of the TCA cycle, which possesses a diverse physiological function from the 'second half' of this pathway. Such diversification in function of various TCA cycle reactions is not without precedence since the cycle was previously demonstrated to display a modular structure in bacteria (Tian, et al., 2005), yeast (McCammon, et al., 2003) and plant systems (Lancien, et al., 1999). Recent studies of Arabidopsis leaves (Kolbe, et al., 2006) seem to confirm this hypothesis. Leaves of these plants responded to DTT treatment by decrease in the levels of organic acids involved in the first part of the TCA cycle (aconitate, isocitrate, and 2-oxoglutarate) and accumulation of intermediates of the second part of the cycle. The authors proposed that control over the TCA cycle was taken by 2-oxoglutarate dehydrogenase complex (OGDC), regulated at the posttranslational level by changes in cell redox status. Similar conclusions were reached, following the recent study of potato tubers treated with specific phosphonate inhibitors of OGDC (Araujo, et al., 2008). The *in vitro* inhibition of the enzyme complex resulted in dramatically limited rate of respiration and alterations in the level of vital C and N metabolites. These data implied that the OGDC complex could be the major regulatory step at the juncture of the TCA cycle and nitrogen assimilation, although such role has previously been assigned to NAD-IDH (Galvez, et al., 1999; Igamberdiev and Gardestrom, 2003). When taken together, it could be expected that inhibition of the enzymes involved in the initial, decarboxylating part of the TCA cycle should result in consistent alterations in plant performance. Unfortunately, to date little 'lack-of-function' research was carried out on these enzymes/genes *in planta*. Among plants exhibiting blockages of CS, Aco and NAD-IDH activity (Landschutze, et al., 1995b; Kruse, et al., 1998; Galvez, et al., 1999; Carrari, et al., 2003a; Lemaitre, et al., 2007) only those carrying lesions in the Aco gene were characterized by enhanced photosynthetic assimilation and transpira-

tion activity. Nevertheless, it must be taken into account that *Lycopersicon pennellii* differs significantly from my cultivated tomato plants as it is a wild species bearing very small and exclusively green fruits (Schauer, et al., 2005). The *Aco* mutant presented additionally mild changes in flowering time and a tendency towards limited number of flowers, similarly to my CS transgenic tomatoes. However the final fruit yield of Aco mutant was largely enhanced in contrast to CS antisensed potato plants, in which flower formation was aborted (Landschutze, et al., 1995b). That said, this comparison reveals some similarities in phenotypic response to partial deactivation of initial steps of TCA cycle in plants, although the magnitude of changes differs dependently on plant species, type of inhibited enzyme and its final remaining activity.

As mentioned above, plants down regulated in the activity of mitochondrially localized CS and NAD-IDH disclosed a few similar physiological and metabolic alterations, which were also typical for other TCA cycle mutants. Interestingly, inhibition of the two analyzed isoforms of isocitrate dehydrogenases resulted in similar response on cellular and whole plant level. As it could perhaps be expected on the basis of the irrefutable genetic, structural and biochemical resemblance between the latter two enzymes, they may play partially similar physiological roles in plants. Indeed, both NAD-IDH and NADP-ICDH displayed alterations in N assimilation pathway, resulting from inhibition in 2-OG production. Moreover, the maximal photosynthetic efficiency was significantly decreased in all IDH/ICDH transgenic lines. These plants also shared similar metabolic profiles, characterized by accumulation of nitrate and decrease in the level of starch, photosynthetic pigments and reduced pyridine nucleotides. In order to balance massive metabolic alterations, both transgenic plants seemed to utilize similar compensatory strategies, involving the up regulation of glutamate dehydrogenase and glycerate kinase activity. Furthermore, the down regulation of either isoform of isocitrate dehydrogenase resulted in a specific phenotype, manifested in strongly decreased both fruit size and fruit yield. The above mentioned alterations seemed to be consistent in both NAD-IDH and NADP-ICDH plants, therefore they could be assigned to limitations in isocitrate dehydrogenase activity *per se*. Moreover, these findings imply that both the cytosolic and mitochondrial isoform of isocitrate dehydrogenase are involved in provision of 2-OG to nitrate assimilation in leaves, therefore their physiological role in tomato metabolism is partially overlapping.

Although the reduction in the activity of either NAD-IDH and NADP-ICDH resulted in some corresponding changes in tomato metabolism, an isoform-specific response was also observed. The down-regulation of cytosolic NADP-ICDH did not cause any major respiratory impediments and it had clear, and much stronger than in the case of the mitochondrial enzyme, negative impact on leaf nitrogen metabolism. The levels of leaf soluble carbohydrates, which was significantly decreased under optimized growth conditions remained lower in ICDH4 line than

in control plants, whereas accumulation of fatty acids was no longer present upon N starvation. Interestingly, the level of 2-oxoglutarate was dramatically decreased to the lowest detection limit of GC-MS and similar reduction was observed for glutamate and aspartate levels. Nevertheless, the remaining TCA cycle intermediates were only marginally, not significantly decreased as compared to both NAD-IDH and wild type plants. Assuming that the activity of other IDH/ICDH isoforms present in various cellular compartments remained unaltered or perhaps even up regulated following the transfer of plants to N deficient conditions, they did not fully compensated for limited NADP-ICDH performance. It could therefore be concluded that in tomato plants the cytosolic NADP-ICDH isoform has little influence on citrate/isocitrate metabolism within mitochondria, however it bears a considerable responsibility for production of 2-OG required for nitrate assimilation. More importantly, the performance of this isoform can be regulated by nitrate level and its function seems indispensable. This finding stays in agreement with current view concerning the physiological role of mitochondrial NAD-IDH isoform that supports respiration by acting within TCA cycle and cytosolic NADP-ICDH isoform providing carbon skeletons for N assimilation pathway (Scheible, et al., 2000; Gray, et al., 2004; Lemaitre and Hodges, 2006; Leterrier, et al., 2007).

According to the above mentioned theory of common regulation of enzymes sharing similar functions in plants metabolism both CS and NADP-ICDH plants should suffer from similar physiological perturbations. Indeed, both sets of plants were characterized by a tendency towards well known symptoms of N deficiency such as increased root formation, whereas NAD-IDH plants presented no specific root phenotype. Such changes combined with an inhibition of the TCA cycle in tomato are not without a precedence, since recently reported dramatic alterations in root weight and root area of the transgenic plants bearing limited activity of mitochondrial malate dehydrogenase and fumarase (Van der Merwe, et al., 2009a). Moreover, both CS and NADP-ICDH tomatoes displayed a mild reduction in fruit yield, however root and fruit phenotype was much more pronounced in NADP-ICDH plants. Apart from the phenotypic alterations, these plants revealed important metabolic changes that manifested limitations in nitrate assimilation pathway. They included accumulation of nitrate and significant reduction in the enzymatic activity of major players in N assimilation metabolism, such as nitrate reductase (both CS and NADP-ICDH) and GS-GOGAT cycle (only CS plants). Moreover, both sets of transgenic tomatoes showed inhibition in enzymes, involved in primary C metabolism, such as PEP carboxylase (both CS and NADP-ICDH) and pyruvate kinase (only NADP-ICDH plants). Interestingly, CS plants were additionally limited in the activity of NADP-ICDH, which may explain the resemblance of the two transgenic genotypes and mask some of the effects of CS inhibition *per se*. The data showed that both CS and NADP-ICDH plants, as opposed to NAD-IDH plants, presented significant limitations in activity of important enzymes required for nitrogen assimilation process. This finding implies that the isoforms may be somehow involved in regulation of nitrogen metabolism in

tomato, which stays in agreement with proposed models of coordination of C and N metabolism in plants (Scheible, et al., 1997a; Stitt, 1999). Additionally to functioning within N metabolism, the cytosolic NADP-ICDH isoform may contribute to the degradation of fatty acids and lipids in glyoxysomes, preceded by the concerted action of peroxisomal CS and cytosolic aconitase. This role was initially proposed by Kruse and coworkers (1998) and was supported by the accumulation of palmitic and stearic fatty acid content and reduction in soluble sugars in my NADP-ICDH tomatoes in contrast to decrease of these fatty acids and accumulation of carbohydrates in CS plants.

Interestingly, the majority of my transgenic plants possessed significantly increased level of glutamine (Gln) and asparagine (Asn), both of which were elevated in all CS and NADP-ICDH lines, whereas Asn was increased in IDH1 line. Moreover, CS transgenic plants exhibited elevated 2-oxoglutarate (2-OG) level in leaves. All these metabolites were suggested to act as sensors of leaf nitrogen status (Glass, et al., 2002; Hodges, 2002; Stitt, et al., 2002; Coruzzi, 2003; Foyer, et al., 2003), although they are also involved in sensing of the availability of C and N source (Ferrario-Mery, et al., 2002b; Noctor, et al., 2002; Novitskaya, et al., 2002; Foyer, et al., 2003; Miller, et al., 2007). The two amino acids are believed to be the major nitrogen forms in plant leaves (Coruzzi and Zhou, 2001; Glass, et al., 2002; Stitt, et al., 2002). Moreover, Gln content is strongly related to NR activity and increases in response to nitrogen supply (Foyer, et al., 1994; Scheible, et al., 1997a), however this correlation was broken after carbon supply. Feeding of 2-oxoglutarate (2-OG) to tobacco leaves resulted in decrease of Gln content, regardless of high NR activity (Muller, et al., 2001). Interestingly, the alteration in 2-oxoglutarate production in my transgenic CS and NADP-ICDH plants resulted in elevation in Gln content and decrease in NR activity. It is believed that key factors determining leaf Gln content are ammonia input and rate of 2-OG supply, and that the level of this amino acids provides information on the balance between leaf N and C metabolism (Foyer, et al., 2003). This view is consistent with results obtained for all my transgenic plants, however best exemplified in CS antisense plants. I hypothesize that glutamine and asparagine, massively accumulated in CS tomatoes, are signaling metabolites carrying information of high N availability in soil, imported from roots to leaves. However, due to the decrease in mitochondrial CS activity followed by diminished performance of TCA cycle the provision of sufficient amount of mitochondrially derived 2-OG molecules for nitrogen assimilation is insufficient. Even if the total cellular demand for carbon skeletons for amino acids synthesis can be partially met, leading to only minor decrease in total amino acid pool and no major phenotypic variations, due to supportive upregulation of peroxisomal CS isoform, the signaling of C source availability is altered as compared to wild type plants. Whether this information is bound to the lowered level of direct products of impaired enzyme or pathway, such as citrate or malate and glucose (as proposed by Seebauer, et al., 2004), or the organic acid standing at the crossroad of C and N metabolism and believed to act as a signaling molecule (Hodges, 2002), namely 2-oxoglutarate, or perhaps another metabolite, is not yet

fully understood. This theory is however supported by steady state amino acids content in CS antisense plants that shows the biggest accumulation within amino acid pool of Asn, which carries more N per C than Gln, therefore is used for transport of nitrogen when levels of carbon are low (Coruzzi, 2003). Interestingly, many aspects of the metabolite profiles of the transgenic CS plants, including the accumulation of Gln and Asn, are reminiscent of those reported during extended night treatment or even during dark-induced senescence (Ishizaki, et al., 2005; Gibon, et al., 2006; Fahnenstich, et al., 2007). Moreover, recent studies performed on common bean have shown that asparagine synthetase is transcriptionally regulated by sugar signaling mechanisms, therefore Asn level is dependent on plant carbon status (Silvente, et al., 2008). Due to the existence of multiple isoforms of aminotransferases the changes in Asn and Gln level are reciprocally dependent. Indeed, concomitant accumulation of both amino acids was found in sulphur-deprived tobacco plants, in which interestingly, it caused a repression of NR transcript (Migge, et al., 2000). Elevation of Gln could also result from massive ammonia release from photorespiratory glycine that exceeds the rate of 2-OG regeneration as glyoxylate availability falls down. An alternative explanation would be that it is influenced by insufficiently reduced Fd for GOGAT activity, that is likely to occur in CS transgenic leaves possessing lowered activity of several enzymes engaged in production of reducing equivalents.

Surprisingly and partially contrary to the hypothesis of Gln content rising at low C availability (Coruzzi, 2003), the level of 2-OG in CS transgenic leaves was not decreased but rather was significantly elevated. One explanation could be that a carbon availability sensing mechanism that subsequently leads to accumulation of Gln is able to distinguish subcellular origin of 2-OG and does not quantify the cytosolic pool of this organic acid, augmented by the combined action of peroxisomal CS and cytosolic aconitase and isocitrate dehydrogenase. Accordingly, the content of 2-OG in plants inhibited in the activity of cytosolic NADP-ICDH isoform was unaltered and resembled wild type level. If this is indeed a case, the carbon sensors could not be located within mitochondria, since neither 2-OG nor Gln levels were significantly changed in my NAD-IDH transgenic plants. However, when taken into account that both CS and NADP-ICDH plants revealed alterations in chloroplastic redox balance, as assumed on the basis of the significantly decreased activation state of NADP-MDH in ICDH3, CS22 and CS25 lines, it could be hypothesized that such sensing mechanism could be located in plastids. This theory is additionally strengthened by the fact that the best known candidate for C:N sensor and coordinator, namely the plant homolog of the bacterial PII protein is equipped with chloroplast targeting peptide and its activity is believed to be restricted to this compartment (Ferrario-Mery, et al., 2005). However, the plant PII protein was shown to sense both energy status in the form of ATP and C source availability as 2-OG, as it synergistically binds to these metabolites, however the covalent modification of PII molecule responding to nitrogen level status in the form of glutamine seems to exist only in *E.coli* and perhaps some other prokaryotes (Smith, et al., 2004b; Jiang and Ninfa, 2007; Mizuno, et al., 2007b).

Although the plant PII protein was proposed to play various physiological roles in plant metabolism (Hsieh, et al., 1998; Ferrario-Mery, et al., 2006; Jiang and Ninfa, 2007; Llacer, et al., 2007; Ferrario-Mery, et al., 2008; Lillo, 2008), the precise mode of action, including the influence on and by 2-OG and Gln level has not yet been elucidated. The explanation I favor is that both plant N and C supply signals in the form of Gln and 2-OG respectively act independently from one another and their effectiveness depends on cell's ability to sense them. Although Gln and 2-OG have been proven to have antagonistic effect on transcript level and activity of some N assimilation pathway enzymes, particularly nitrate reductase (Dzuibany, et al., 1998; Ferrario-Mery, et al., 2001), the concomitant changes in their level, such as the one I observed in CS transgenics are not without a precedence. Tobacco lines suffering from restriction in nitrate assimilation caused by inhibition of Fd-GOGAT activities to a wide range, were characterized by marked increase in both Gln and 2-OG (Ferrario-Mery, et al., 2000). The nocturnal accumulation of Asn in these transgenic plants pointed in an important role of this amino acid as a temporary storage compound for the elimination of excess photorespiratory ammonia (Ferrario-Mery, et al., 2002a), that would explain this phenomenon in CS, NAD-IDH and NADP-ICDH antisense plants, characterized by elevated photorespiratory flux.

The purpose of this work was to investigate the influence of the three major enzymes of central metabolism on plant physiology and performance, with special focus on illuminated leaves. This is the first report of cloning and functional analysis of mitochondrial CS, NAD-IDH and cytosolic NADP-ICDH enzymes in plants of such vital agronomic importance as tomato. The detailed analysis of transgenic plants down regulated in the activity of two rate-limiting TCA cycle enzymes, that is CS and NAD-IDH revealed not only a specific metabolic and transcript response towards inhibition of selected gene in tomato but also a handful of symptoms common for other TCA cycle mutants. They included alterations in respiration ability and inhibition in flux through the TCA cycle. Interestingly, all my transgenic tomato plants were characterized by accumulation of nitrate, in addition to displaying various symptoms of limitations in N assimilation pathway. That finding was not particularly surprising for NADP-ICDH, which was previously proposed to serve as a carbon donor for GS-GOGAT cycle (Chen and Gadal, 1990; Fieuw, et al., 1995; Scheible, et al., 1997a; Galvez, et al., 1999; Stitt, 1999; Scheible, et al., 2000; Igamberdiev and Kleczkowski, 2001; Lemaitre and Hodges, 2006). Indeed, my data showed that this isoform had little effect on the mitochondrial respiration in tomato plants, although it may be the main supplier of 2-OG residue for nitrate assimilation. These results are in accordance with current understanding of regulation and signaling of various C and N level in plants (Hodges, 2002; Stitt, et al., 2002; Coruzzi, 2003; Foyer, et al., 2003). Moreover, the results obtained for CS and NAD-IDH antisense plants suggested that in tomato TCA cycle plays an important, additional to respiration, role in regulation of supply of organic acids for N assimilation. Such function was previously assigned to mitochondrial NAD-IDH isoform (Lancien, et al., 1999; Stitt and Fernie, 2003;

Abiko, et al., 2005b). My data therefore confirm the profound impact that mitochondrially localised proteins have on nitrogen metabolism in plants (Dutilleul, et al., 2005; Pellny, et al., 2008). Furthermore, although none of my transgenic plants were inhibited in carbon assimilation, they all revealed decrease in photosynthetic pigments. That said, my data hinted into a close collaboration between mitochondrial and chloroplastic metabolism and stay in agreement with previous reports of TCA cycle mutants possessing altered photosynthetic performance (Carrari, et al., 2003a; Nunes-Nesi, et al., 2005b; Nunes-Nesi, et al., 2007a).

The results presented here provided a deeper insight into of the functional role of mitochondrial citrate synthase and mitochondrial and cytosolic isocitrate dehydrogenases in leaf metabolism of a model plant (*Solanum lycopersicum*). They highlighted the existence of interorganellar coordination of metabolism and increased our understanding of carbon-nitrogen interactions. Additionally, they proven the presence of strategies, by which metabolism is reprogrammed to compensate for the emerging deficiencies.

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Curriculum Vitae

List of publications

Agata Sienkiewicz-Porzucek, Ronan Sulpice, Sonia Osorio, Ina Krahnert, Andrea Leisse, Ewa Urbanczyk-Wochniak, Michael Hodges, Alisdair R. Fernie, Adriano Nunes-Nesi (2009)
Mild reductions in mitochondrial isocitrate dehydrogenase activity result in compromised nitrate assimilation, pigmentation and maximum photosynthetic efficiency but do not impact growth.
(accepted by Molecular Plant in 2009)

Agata Sienkiewicz-Porzucek, Adriano Nunes-Nesi, Ronan Sulpice, Jan Lisec, Danilo C Centeno, Petronia Carillo, Andrea Leisse, Ewa Urbanczyk-Wochniak, Alisdair R. Fernie (2008)
Mild reductions in mitochondrial citrate synthase activities result in a compromised nitrate assimilation and reduced leaf pigmentation but have no effect on photosynthetic performance or growth.
(Plant Physiology 147, 115-127)

Megan Morgan, Martin Lehmann, Markus Schwarzlander, Charles J Baxter, Agata Sienkiewicz-Porzucek, Thomas C.R. Williams, Nicolas Schauer, Alisdair R Fernie, Mark D Fricker, R George Ratcliffe, Lee Sweetlove, and Iris Finkemeier (2008)
Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. (Plant Physiology 147, 101-114)

Agata Sienkiewicz-Porzucek, Berlin, September 2009

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